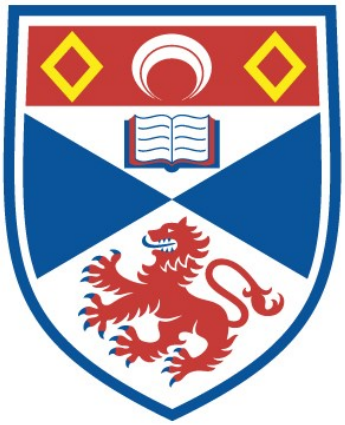
THE ROLE OF AUXIN AND CYTOKININ IN THE CONTROL OF MORPHOGENESIS IN VITRO IN THE PLANT FAMILIES 'ROSACEAE' AND 'ERICACEAE'

Margaret E. Norton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE ROLE OF AUXIN AND CYTOKININ IN THE CONTROL OF MORPHOGENESIS IN VITRO IN THE PLANT FAMILIES ROSACEAE AND ERICACEAE

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Declaration

I hereby declare that the following thesis is based upon work done by me, that the thesis is my own composition and that it has not previously been presented for a higher degree.

The research was carried out in the Department of Botany of the University of St.Andrews under the supervision of Dr. D. C. Weeks, and in the Department of Plant and Soil Sciences, University of Idaho, U.S.A. under the local supervision of Dr. A. A. Boe and Dr. L. Calpouzos.

Certificate

I hereby certify that Margaret E. Norton has been engaged upon research work for a minimum of nine terms under my supervision, that she has fulfilled the conditions of Ordinance Number Twelve, and that she is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Acknowledgements

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At the University of St. Andrews, I would like to record my thanks to Professor D.H.N. Spence and Professor J.A. MacDonald in whose department I worked. My sincere thanks goes to Dr. D.C. Weeks for supervising my work and making this work possible.

I should also like to thank my husband, Colin, for his encouragement.

ABSTRACT

The regulation of morphogenesis by exogenous auxin / cytokinin ratio was investigated in thirty ornamental species and cultivars of Rosaceae and Ericaceae. Shoot explants were cultured *in vitro* on nutrient medium containing 0 to 20 mg l^{-1} indolebutyric acid (IBA), naphthalene-acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA), isopentenyladenine (2iP), or kinetin. Shoot, root and callus formation were recorded after four-week incubation periods. Exogenous cytokinin was necessary for shoot formation, and exogenous auxin for root formation, while callus formation occurred in the presence of exogenous auxin or cytokinin.

BA induced the formation of many short shoots whereas 2iP induced fewer longer shoots probably due to 2iP oxidation and BA-stimulated ethene synthesis. Four different callus types formed in response to growth regulator applied, growth regulator concentration, light and temperature. Auxin-induced callus and root formation was promoted by darkness whereas cytokinin-promoted callus formation was promoted by light.

Elimination of potassium from the medium increased root formation but decreased shoot and callus formation. Low medium pH promoted root and callus formation while a higher pH stimulated shoot formation. The presence of axillary buds and the shoot apex promoted root and callus formation but inhibited shoot formation.

Cytokinin-enhanced respiration rate and ethene synthesis was related to caulogenesis, but auxin-promoted respiration rate and ethene synthesis was not correlated with rhizogenesis. Ethephon and ethene promoted simultaneous shoot and root formation but

ethene was shown to be unlikely to act as an intermediate in all auxin- and cytokinin- induced differentiation.

Repeated subculture of shoots resulted in an initial rise followed by a decline in caulogenesis, a decline in root formation and an increase in callus formation (apparently irreversible decline). Shoot length and leaf size decreased with repeated subculture but increased after auxin, gibberellin, or decreased illumination treatment.

Auxin and cytokinin treatment could not override species and cultivar variation in morphogenesis.

CONTENTS

I. INTRODUCTION

The hypothesis of regulation of morphogenesis in plants by auxin / cytokinin ratio (Skoog and Miller, 1957) has been widely accepted (e.g. Hussey, 1978). This hypothesis based on the tobacco callus system of Skoog and Miller has been verified for tobacco (Vasil and Hildebrandt, 1965; Van et al., 1974) and other species have also been shown to conform to this concept (e.g. Petunia - Durand et al., 1973). Both auxin and cytokinin are believed to be essential in the regulation of morphogenesis, while the direction of development is determined by their concentration. Thus, a high cytokinin / auxin ratio promotes shoot initiation, a high auxin / cytokinin ratio promotes root initiation, and intermediate levels of both auxin and cytokinin lead to callus growth in cultured tissues.

However, morphogenesis in some species does not appear to be regulated by auxin / cytokinin ratios. For example, a high cytokinin / auxin ratio favoured root formation and a high auxin / cytokinin ratio promoted shoot initiation in alfalfa callus (Walker et al., 1978); transfer of thin cell layers of Nicotiana to an auxin-free medium promoted morphogenesis (Tran Thanh Van and Cousson, 1980); and regeneration of

shoots and roots from callus in many woody plant species has not been accomplished by adjusting the exogenous auxin / cytokinin balance (Murashige, 1977a).

This variability may be accounted for in part by interaction of endogenous auxin and cytokinin with the exogenous supply, and failure to regenerate shoots and roots may indicate limitation by other factors, for example, environmental or nutritional factors (Murashige, 1977a). However, even when conditions satisfactory for growth are met, many species still fail to respond to auxin and cytokinin application by changing morphogenetic development.

A further example of inability to respond to auxin and cytokinin has been noted in long-term callus and cell suspension cultures. Potential for differentiation was impaired after lengthy periods in culture (Torrey, 1967; Smith and Street, 1974). In these cases, sensitivity to auxin and cytokinin is reduced with time in culture, even when conditions previously conducive to morphogenesis were maintained.

The above examples demonstrate that regulation of morphogenesis by auxin and cytokinin concentration is not always possible. The theory of regulation by concentration has been challenged : plant growth

regulators act over a wide concentration range and therefore, although their presence may be essential, it has been proposed that they are unlikely to control growth and differentiation by concentration (Trewavas, 1980).

Previous work has not attempted to relate results from callus formation experiments to results from differentiation experiments in the same species. Species differences in potential for differentiation and in growth regulator activity have frequently been reported (Linsmaier and Skoog, 1965) and therefore, it is only by examining the same species under the same conditions that we can gain a meaningful understanding of the control of dedifferentiation and differentiation.

Few studies of morphogenesis in vitro have taken into account the genetic background of the plant. Linsmaier and Skoog (1965) found that five tobacco cultivars varied in their capacity to produce shoots and Bingham et al. (1975) demonstrated a difference in morphogenetic potential between alfalfa cultivars. However, no comprehensive studies have been made to determine the extent of similarities and differences between genera, species and cultivars.

Little comparative work has been conducted on the families Rosaceae and Ericaceae in vitro. Anderson (1975, 1978a) has worked on adventitious shoot formation and subsequent rooting of shoots in Rhododendron, Robenek (1979) studied growth of Rhododendron callus and Nickerson and Hall (1976), Nickerson (1980) and Zimmerman and Broome (1980) have worked on Vaccinium. In Rosaceae, much research has focussed on fruit cultivars, for example, apple (Abbott and Whiteley, 1976; Jones et al., 1977) and Prunus (Boxus, 1975; Jones and Hopgood, 1979). These studies, although very useful from a practical standpoint, concentrate on development of propagation techniques rather than on reasons for the observed effects.

Application of exogenous auxin and cytokinin frequently results not only in a change in morphogenetic pattern but also in changes in endogenous growth regulator concentrations and in metabolism. Ethene concentration changes after auxin or cytokinin application (Morgan and Hall, 1962, 1964; Lau and Yang, 1973) - ethene levels rise as auxin and cytokinin concentrations increase. Ethene has been shown to be involved in rhizogenesis in stem cuttings (Kawase, 1971), in callus formation (Goren et al., 1979), and

auxin-induced ethene production plays a role in inhibition of root elongation. Ethene, therefore, could be a mediating compound regulating morphogenetic response to auxin and cytokinin.

Respiration rate has been demonstrated to either increase after auxin or cytokinin application (Bonner, 1933; Moore and Miller, 1972) or decrease (Humphreys and Dugger, 1957; Tetley and Thimann, 1974). The effect of growth regulator concentration on respiration is unclear. It is hypothesised that respiration rate is either increased or decreased depending on the concentration of auxin or cytokinin applied. Change in respiration rate may occur either before morphogenesis or as a consequence of increased growth due to morphogenesis.

This thesis investigates (1) auxin and cytokinin induced morphogenesis in excised shoots and seeks evidence to support or negate the hypothesis of growth regulator control by concentration, (2) change in auxin and cytokinin sensitivity in long term cultures of shoot explants - shoots are already highly differentiated unlike the callus used by others for such experiments and therefore are less likely to lose their capacity for further differentiation, (3)

differences in control factors for dedifferentiation and differentiation, (4) the role of ethene and respiration in mediating morphogenetic response to auxin and cytokinin concentration, and (5) all of the above in a large number of species of the families Rosaceae and Ericaceae.

The species chosen for the current work are important as ornamental plants and therefore, this work, in addition to investigating the control of morphogenesis, has practical application in terms of commercial micropropagation.

II. MATERIALS AND METHODS

2.1 SPECIES USED IN THE EXPERIMENTS

The following species and cultivars were used in the experiments:-

Ericaceae

Arctostaphylos media Greene

A. uva-ursi (L.) K. Spreng

Erica carnea L. cv. Springwood White

Gaultheria hispidula (L.) Muhlenb. ex Bigel

Kalmia angustifolia L. (pink form)

Rhododendron arboreum Sm.

R. chamae-thomsonii (Tagg & Forr.) Cowan & Davidian

R. hyb. Chikor

R. hyb. Chinsayii

R. concinnum Hemsl.

R. dauricum L.

R. fastigiatum Franch.

R. forrestii Balf. f. ex Diels

R. keiskei Miq.

R. leucaspis Tagg

R. lutescens Franch.

R. hyb. P. J. M. Victor

R. ponticum

R. racemosum Franch.

R. hyb. Vuyk's rosy red

R. williamsianum Rehd. & Wils.

Vaccinium vitis-idaea var. minus Lodd.

Rosaceae

Chaenomeles japonica (Thunb.) Spach

Cotoneaster dammeri Schneid. cv. Mooncreeper

Crataegus brachyacantha Sarg. & Engelm.

Crataegus X mordenensis Boom. cv. Toba

Malus hyb. Dainty

Malus hyb. Golden Hornet

Potentilla fruticosa L. cv. Coronation Triumph

Potentilla fruticosa L. cv. Sutter's Gold

Prunus cerasifera Ehrh. cv. Thundercloud

Prunus tomentosa Thunb.

Pyracantha coccinea Roem. cv. Lalandei

Spiraea X bumalda Burv. cv. Froebelii

Species and cultivar selection maximized the range of difficulty of vegetative propagation in the families Ericaceae and Rosaceae. Sources of the plants used are listed in Appendix 11.1.

2.2 TECHNIQUES USED FOR IN VITRO CULTURE

2.21 Composition of nutrient medium

Three published nutrient formulations were used in the experiments :- 1) Linsmaier and Skoog (1965) (abbreviated to LS), 2) Anderson (1975) (abbreviated to A1) and 3) Anderson (1978b) (abbreviated to A2). Table 1 gives the composition of these media. The Anderson media, formulated for the culture of Rhododendron hybrids, are similar to the medium of Murashige and Skoog (1962) (abbreviated to MS) and the later revision from the same laboratory (Linsmaier and Skoog, 1965) both of which were formulated for the culture of tobacco callus.

Anderson's medium (1975) has a reduced concentration of potassium - 950 mg l^{-1} KNO_3 in place of the 1900 mg l^{-1} of the Linsmaier and Skoog formulation. Anderson's media were developed after observation of explant browning and early death of Rhododendron explants when cultured on Murashige and Skoog medium. Reduction of KNO_3 and NH_4 NO_3 concentration reduced these problems (Anderson, 1978b). Anderson showed that NaH_2 PO_4 $.H_2$ O was beneficial to the growth of

Rhododendron cultures and that adenine sulphate was important in stimulating growth of axillary buds and to sustain shoot growth. Anderson's medium was used in the current work for the culture of all Ericaceous species except Rhododendron concinnum in which A1 Anderson medium was used (experiments conducted prior to development of A2 medium). Murashige and Skoog (1962) salts are satisfactory for the culture of plants from the family Rosaceae (Jones and Hopgood, 1979) and were used for the culture of all Rosaceous species in the current experiments (identical salt composition to LS).

Sucrose is used as a carbon source in the culture of a diverse range of species and has proved satisfactory for most species. Coffin et al. (1976) studied callus growth in species of Rosaceae after sorbitol substitution for sucrose. Callus growth decreased in all species tested, except Prunus persica. Sucrose was therefore used as the carbon source in these experiments.

Myo-inositol enhances callus growth in tobacco (Linsmaier and Skoog, 1965; Gamborg et al., 1976), promotes bud formation in cultures of Ulmus (Jacquiot, 1966) and promotes embryogenesis in carrot

(Norreel and Nitsch, 1968). Myo-inositol is routinely incorporated in most culture media (Murashige, 1973b) and was used in all media in the following experiments.

Linsmaier and Skoog (1965) concluded that for tobacco callus culture, the vitamin thiamine was essential but that neither pyridoxine nor nicotinic acid (included in MS medium) were beneficial and could retard growth. Amino acids, such as glycine, have also been shown to be non-essential constituents of the nutrient medium (Linsmaier and Skoog, 1965) and are not normally incorporated in media for either callus or shoot culture (Murashige, 1973b). Therefore, in the following experiments, thiamine was added to all media but no other vitamins or amino acids were used.

N.B. In papers by Murashige and Skoog (1962) and Linsmaier and Skoog (1965), the quantity of $ZnSO_4.4H_2O$ used was listed as 8.6 mg l^{-1} which gave a concentration of 30μM. This statement is incorrect due to error from degree of hydration of $ZnSO_4$ thus 8.6 mg l^{-1} $ZnSO_4.7H_2O$ gives a concentration of 30μM.

Table 1.

Nutrient media formulations used in the experiments (mg l^{-1})

	LS	A1	A2
Inorganic salts			
NH_4NO_3	1650	2000	400
KNO_3	1900	950	480
$CaCl_2 . 2H_2O$	440	440	440
$MgSO_4 . 7H_2O$	370	370	370
KH_2PO_4	170	170	-
$NaH_2PO_4 . H_2O$	-	170	380
Na_2 EDTA	37.3	37.3	74.5
$FeSO_4 . 7H_2O$	27.8	27.8	55.7
H_3BO_3	6.2	6.2	6.2
$MnSO_4 . 4H_2O$	22.3	-	-
$MnSO_4 . H_2O$	-	16.9	16.9
$ZnSO_4 . 7H_2O$	8.6	8.6	8.6
KI	0.83	0.83	0.83
$Na_2MoO_4 . 2H_2O$	0.25	0.25	0.25
$CuSO_4 . 5H_2O$	0.025	0.025	0.025
$CoCl_2 . 6H_2O$	0.025	0.025	0.025
Organic constituents			
thiamine.HCl	0.4	0.4	0.4
adenine sulphate. dihydrate	-	80	80
myo-inositol	100	100	100
sucrose	30000	30000	30000

2.22 Use of growth regulators

The following growth regulators were used in the experiments:-

auxins

IBA indole-3-butyric acid

NAA naphthaleneacetic acid

2,4-D 2,4-dichlorophenoxyacetic acid

cytokinins

BA N_6 -benzyladenine or N_6 -benzylaminopurine

2iP N_6 -isopentenyladenine or 6-(gamma, gamma,-dimethylallylamino) purine

Kn kinetin

GA gibberellic acid (GA_3)

Concentrations used ranged from 0 to 50 mg l^{-1}. These compounds have a low solubility in water and therefore were dissolved in 5 ml of either 1N KOH or 1N HCl prior to making up to the required volume with distilled water. Growth regulator solutions were

stored in darkness under refrigeration for a maximum of two weeks.

2.23 Gelation of medium

When a solid medium was required, Difco Bacto agar was added to the medium at a concentration of 7g l^{-1} unless otherwise stated. In experiments where a range of medium acidities was used, different concentrations of agar were used as described in individual experiments. In alkaline media, 0.7% agar may result in high viscosity gel (see Appendix 11.2). An excessively hard gel can inhibit growth of plant tissue (Murashige, 1977a).

The use of an agar medium is not always satisfactory, as agar contains small quantities of impurities. In Difco Bacto agar, 285ppm magnesium may be present, in addition to lesser concentrations of calcium, barium, silica, chloride, sulphate, nitrogen, iron and copper (Pierik, 1971). Growth regulators and toxic substances may also be present. These may affect growth and survival of the explant (Romberger and Tabor, 1971) and affect the conclusions drawn from

nutritional experiments.

When the medium is solid, only a small part of the explant is in contact with the medium with the result that gradients of nutrients, growth regulators, toxic waste products, and gases occur after a short period of culture (Yeoman, 1973). Diffusion rates of such substances depend on agar concentration (Romberger and Tabor, 1971).

For the above reasons, a liquid medium was used in some experiments. However, conditions of liquid culture can be partially anaerobic (Murashige, 1977a), and therefore, to ensure that the explant would receive sufficient oxygen, culture vessels were either agitated, (in a shaker bath), or the plant tissue was supported above the medium on a filter paper support similar to that used by Heller (1973). The filter paper acted as a carrier for the liquid nutrient medium (Plate 3).

2.24 Preparation of nutrient medium

Stock solutions of nutrients were prepared as follows:-

1) major salt concentration X 10

2) minor salt concentration X 200

3) thiamine X 50

4) Fe.EDTA X 100

The required volumes were then combined and diluted as required. Stock solutions were stored in darkness at 4°C for a period not exceeding four weeks. Other constituents were freshly prepared for each batch of medium and used the same day.

When agar was used, it was added to the medium and heated until the agar dissolved. It was then poured into culture vessels.

The pH of the medium was adjusted to 5.8 for Rosaceae and to 4.8 for Ericaceae prior to autoclaving by the addition of 1N HCl or 1N KOH. In some experiments, a pH less than 4.8 was required. In this case, the pH was adjusted after autoclaving because agar does not gel adequately at low pH (Murashige, 1973a) (see Appendix 11.2 for results of experiments on pH and agar gelation).

2.25 Culture vessels

The culture vessels used were culture tubes (25 X 150 mm) except for experiments with Rhododendron concinnum which utilised Erlenmeyer flasks (150 ml)(conducted in a different laboratory). 25 ml of medium was dispensed to each vessel. The vessels were plugged with non-absorbent cotton wool and capped with either polypropylene tube caps or Parafilm (TM). In both cases, the caps allowed some filtered gas exchange to take place.

2.26 Sterilization of nutrient medium

The nutrient medium was sterilized by autoclaving in the culture vessels at 121° C and 15 lb in^2 for fifteen minutes.

All growth regulators, with the exception of GA, were added to the medium before autoclaving. Bragt and Pierik (1971) showed that autoclaving reduced the gibberellin activity of GA by more than 90%. In the current experiments, gibberellin was sterilized by filtration through Millipore (TM) filters with a pore diameter of 0.22 μm, and added to the medium after autoclaving when the medium had cooled to 35° C.

Autoclaving does not destroy the auxins used (Posthumus, 1971), ABA (Wilmar and Doornbos, 1971) or kinetin and N_6 -substituted purines (Dekhuizen, 1971).

Sucrose was also added to the medium prior to autoclaving. Sucrose can be hydrolysed during autoclaving, particularly when incorporated in nutrient media (Ferguson et al., 1958), but it has been shown that autoclaved sucrose has an increased promotional effect on growth of plant tissue after autoclaving (Maretzki et al., 1974).

2.27 Surface sterilization of explants

Shoot tips (15mm in length) were selected from field or greenhouse grown plants (Plate 4). The volume of explant was in some cases critical to survival (see Appendix 11.3). Shoot tips were surface sterilized after leaf removal by immersion in a 0.5% sodium hypochlorite solution or a 10% Clorox bleach solution (0.5% sodium hypochlorite as active ingredient) for ten to thirty minutes (time dependent on condition of explants - see Appendix 11.4). To aid wetting, a few

drops of detergent were added to the solution. On removal from the sterilizing solution, shoot tips were rinsed twice in sterile distilled water.

Unless otherwise stated, explants were apical sections, including the terminal bud, of actively growing shoots with little secondary growth (Plate 4). Explants from older shoots required a longer surface sterilization treatment and frequently became brown on the exterior within a few hours of excision from the parent plant. When such explants were used, they were treated with antioxidants prior to sterilization. This treatment reduced browning (see Appendix 11.5). The best treatment was determined to be a one hour soak in a solution of ascorbic acid (100mg l^{-1}) and citric acid (150 mg l^{-1}).

2.28 Sterile transfer of explants to culture vessels

Sterile transfer of explants and subculturing was accomplished using either a laminar flow cabinet or a sterile room. The fan of the laminar flow hood was turned on for 20 minutes prior to use, and the working area was washed with 70% ethanol. The sterile room was

sterilized with the use of a bactericidal ultra-violet lamp and also by swabbing with ethanol.

Instruments used in the transfer process were sterilized by immersion in 70% ethanol and flaming. They were briefly dipped in cold sterile distilled water prior to handling explants. The openings of flasks and tubes were briefly flamed prior to closure. One explant was placed in each tube or flask. Explants were laid horizontally on the surface of solid media(Plate 2). They were embedded to a depth of approximately one third of their thickness.

2.29 Incubation of cultures

Culture tubes were slanted at an angle of 20° from horizontal. Temperature was maintained at 25° C +/- 2° C unless otherwise stated. Cool-white fluorescent light at 73$\mu \epsilon$ $\bar{m}^2$ s^{-1} was provided (at explant level) for 16 hours per day except for experiments with Rhododendron concinnum which used cool-white fluorescent light supplemented by tungsten bulbs (total illumination = 110$\mu \epsilon$ m^{-2} s^{-1}) (different laboratory). Lights were placed above the

culture vessels (Plates 1 and 2).

2.3 METHODS USED FOR GREENHOUSE EXPERIMENTS ON RHIZOGENESIS

2.31 Preparation of plant material

Softwood tip cuttings of lateral shoots were used in all cases unless otherwise stated. Cuttings were excised from the parent plant using a knife sterilized with 70% ethanol. This minimized entry of pathogens into the vascular tissue. Basal leaves were removed by hand but the cuttings were not otherwise wounded. The length of the cuttings varied with species but included at least three nodes above the level of the medium and two nodes below the surface of the medium.

When cuttings were treated with auxin, they were treated with a quick dip (IBA - 0.3%) for 5 seconds. The quick dip solution was prepared by dissolving 300 mg IBA in 100 ml of 50% ethanol (Hartmann and Kester, 1975). Cuttings were dipped in solutions to a depth of approximately two times the diameter of the stem.

2.32 Rooting medium

The rooting medium consisted of coarse grade peat

and coarse perlite (in a ratio of 1:1 by volume). A coarse medium was used to maximize the air space in the medium because oxygen has been shown to improve rooting of cuttings (Howard, 1976). The use of a coarse medium also decreases waterlogging and conditions which lead to proliferation of fungal disease organisms.

The medium was contained in seed trays (17.0 X 10.8 cm). The cuttings were spaced to avoid foliage contact in order to minimize possible transfer of metabolites and toxic waste substances from one cutting to another.

2.33 Environmental conditions for rooting of cuttings

Trays of cuttings were placed under a clear polythene cover supported by a low wooden frame. This maintained high humidity around the cuttings. Temperature was maintained at approximately 25° C with fluctuations normally experienced in a greenhouse. No basal heat was given. Cuttings taken on short winter days received supplementary illumination for 16 hours per day.

2.4 RESPIROMETRY

Respiration was measured using a Gilson differential respirometer (constant pressure). The entire piece of plant tissue from one culture tube was placed in one respirometer flask for measurement. Gas exchange was measured over a period of two hours after an initial stabilization period.

Oxygen uptake was measured by decrease in volume in flasks containing 2 ml KOH (10%) in the centre well, while net gas exchange was measured in flasks without KOH. All flasks contained 2 ml distilled water to prevent the plant material from drying out and to maintain normal metabolism. Glass beads were added to to prevent submergence of explants.

Carbon dioxide evolution was estimated by difference calculation. Results are given as μl g dry weight plant tissue. The plant samples were dried in an oven at 60°C for 24 hours prior to weighing.

2.5 ETHENE ASSAY

Ethene synthesis was estimated indirectly by measuring the concentration of ethene in culture vessel atmosphere after incubation of plant material in tightly capped culture tubes. Incubation periods are recorded for each experiment. The method used assesses only ethene which diffuses from the tissue into the tube, and does not take account of ethene present in intra- or inter-cellular air spaces in the tissue, or that which may be present in solution within the tissue. Higher values for ethene concentration have been recorded using techniques for extracting ethene from the plant tissue, for example, by subjecting the tissue to vacuum below the surface of a solution in which ethene is insoluble (Beyer and Morgan, 1970). However, such methods were not employed here, as it is possible that during the vacuuming process, wound ethene is produced, thus contributing to the high concentration recorded (Ward et al., 1978). Also, these methods cause waterlogging of the tissue which cannot then be assayed again at a later stage. (ie for sequential studies). Some of the experiments conducted here required that repeated measurements be made at

intervals over a time period.

Plant material was incubated in culture tubes capped with rubber caps. These did not allow gas exchange between the outside air and the gas inside the tube as did the closures used during normal culture of explants. Rubber gives off ethene under certain conditions (Ward et al., 1978), and therefore, a) rubber caps were left open to the air for several days prior to use, b) rubber caps were sterilized with absolute ethanol and c) empty tubes (no plant tissue) were set up simultaneously and checked for the presence of ethene at the end of the incubation period. These tubes acted as a check for ethene present but not synthesised by plant material.

Ethene in the tubes was assayed by withdrawing gas samples from the tubes with a syringe inserted through the rubber cap. Gas samples from the tubes (0.5 ml) were analysed using a Fisher/Victoreen Series 4400 gas chromatograph with flame ionization detection. The column (Applied Science Labs. Inc.) was 1/8 inch X 2 m packed with alumina 60/80 mesh and maintained at 100° C. Results were recorded on a Fisher Recordall Series 5000recorder calibrated with known standards (0.5ml of 5ppm and 10ppm ethene standards).

Dry weight of the tissue was obtained after the ethene levels had been determined. Ethene (ppm) per gram dry weight of explant tissue was calculated.

2.6 DATA ANALYSIS

Regression models were developed for dose-response or time course response relationships. These models fulfilled two purposes a) as deterministic models, and b) as descriptive models. A polynomial model was used in most instances. Fit was determined using a high order model, decrementing to lowest order giving a good fit. For a given set of studies, models of the same order were used even if this resulted in over-fit for some models. Evidence of over-fit is given at the extremes in those instances, but this does not affect the assumptions drawn from the experiments.

The general linear model was used for all regression analyses including polynomial analyses. A coefficient of determination (R^2) states the proportion of total variability accounted for by y, while a correlation coefficient (r) gives a measure of linear fit (also used for polynomial fit).

Modelling also facilitated the use of several transformations for both x and y. The use of transformations was supported by Hartley's test for homogeneity. The most frequent transformation adopted was a $\log_e$ transformation of x data (x + 1

transformation).

Wide ranges were used for independent axis intervals in many experiments. A spline regression could give a more accurate representation of this data. However, the increased precision of fit is not necessary.

A computer program was written to prepare the plots and derive data, while the program used an iterative technique to optimal x range response.

Regression coefficients were compared for several models where a linear relationship was demonstarted. An appropriate test was adopted depending upon whether the unknown residual variances were assumed equal or not.

Some of the data generated was enumerative. Several approaches were adopted. Use of multinomial experimental methods was frequent when sample numbers were small (for χ^2 only if expected number was greater than 5). Pooling of data classes was necessary in some instances, to satisfy the criteria for the multinomial analysis used. An exact probability test was developed for experiments where a response such as survival percentage was compared for two treatments. This test was identical to the exact test for contingency tables

where the event observed and the probability of all more extreme events summated.

Some data sets were transformed before conventional analyses. Hartley's test of homogeneity suggested the necessity for this in some cases. The most frequently used transformations were :- (1) Where the variance equals mean of y, the transformation $\sqrt{y}$ stabilized the variance. Where means are less than 5, a $\sqrt{y + .375}$ transformation was used. (2) Where variance of y equals mean of y^2, a logarithmic base e transformation was used. (3) Where data were recorded as proportions or percentages, an arcsine $\sqrt{y}$ transformation was used ($\sin^{-1}\sqrt{y}$). In this instance, data was transformed arbitrarily to a proportion in some experiments.

In some instances, the transformations are reported in the text. It should be emphasised that these transformations did not dramatically change the conclusions drawn from the experiments, as most analyses yielded similar conclusions. Where the similarity was great, non-transformed data has been presented to aid clarity even although transformation was technically more appropriate.

Standard experimental designs were used for most

of the experiments. Frequently, facilities available determined the adoption of a completely randomized design. Care was taken to ensur a sufficiently large number of degrees of freedom to partition for all appropriate interactions in these analyses. Results are presented as tables of analyses of variance (AOV) with associated probabilities based on a F test. Where these AOV tables are not supported by graphical representation of data or data tables, then a table of main effects (means) is presented. Two methods are used for multiple comparisons. Firstly Fisher's least significant difference test (LSD) has been used and appended to the table of means. Secondly, where highlighting of individual mean separations was desirable, a Duncan's multiple range test has been adopted in lieu of Fisher's LSD. In both of these instances, an α level (for type one errors) of $p = 0.05$ has been adopted. The lower case alphanumeric designation is used for Duncan's mean separation. Lower levels of probability have not been identified (for reasons of clarity) although the level of significance is frequently higher than the rejection region specified.

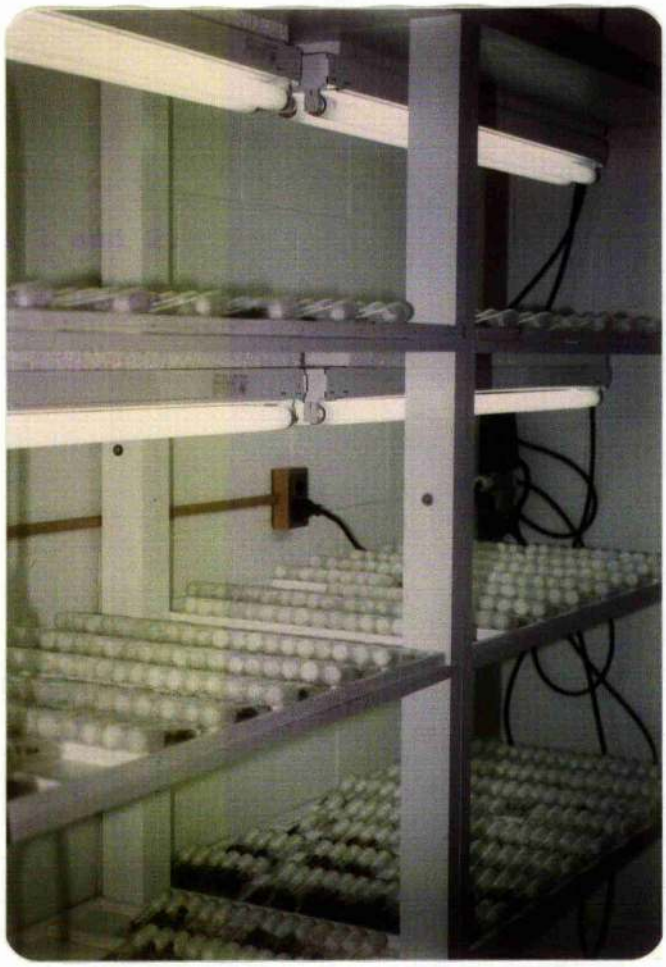
Comparison of small sample means was usually based

on a t test, assuming a normal population distribution (in some instances after appropriate transformation).

Plates 1 and 2.

Cultures under

incubation



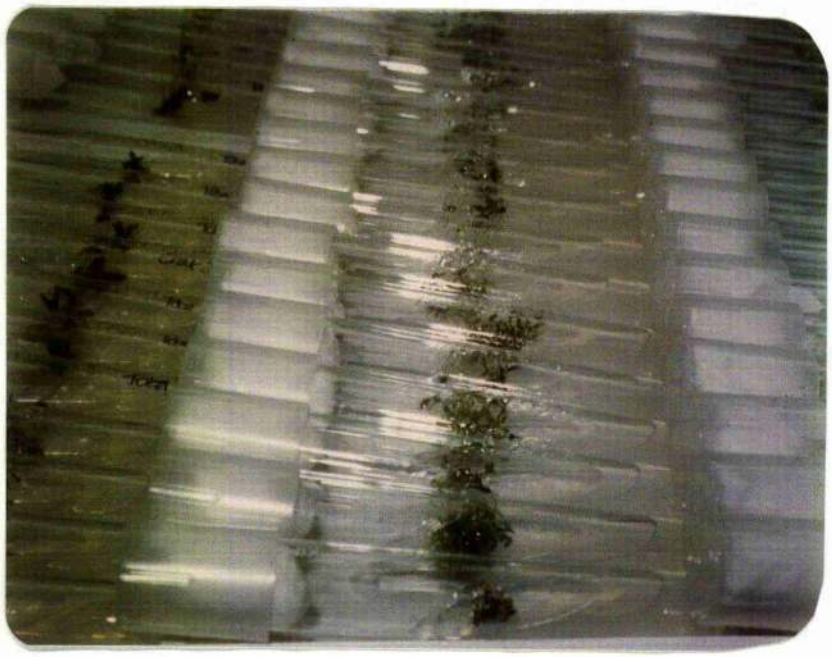
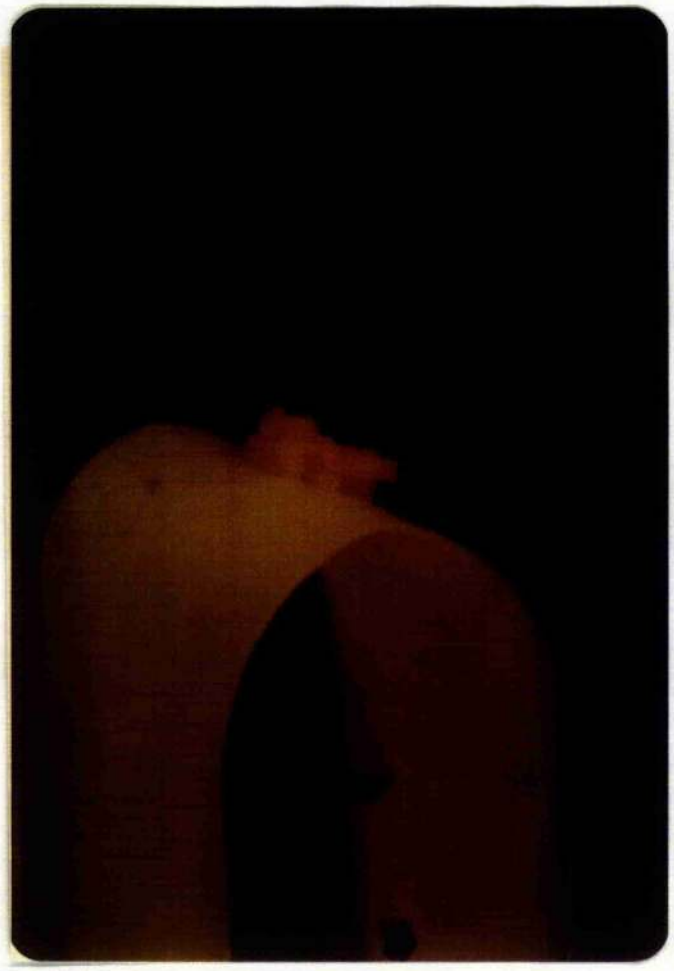


Plate 3.

Filter paper support for callus



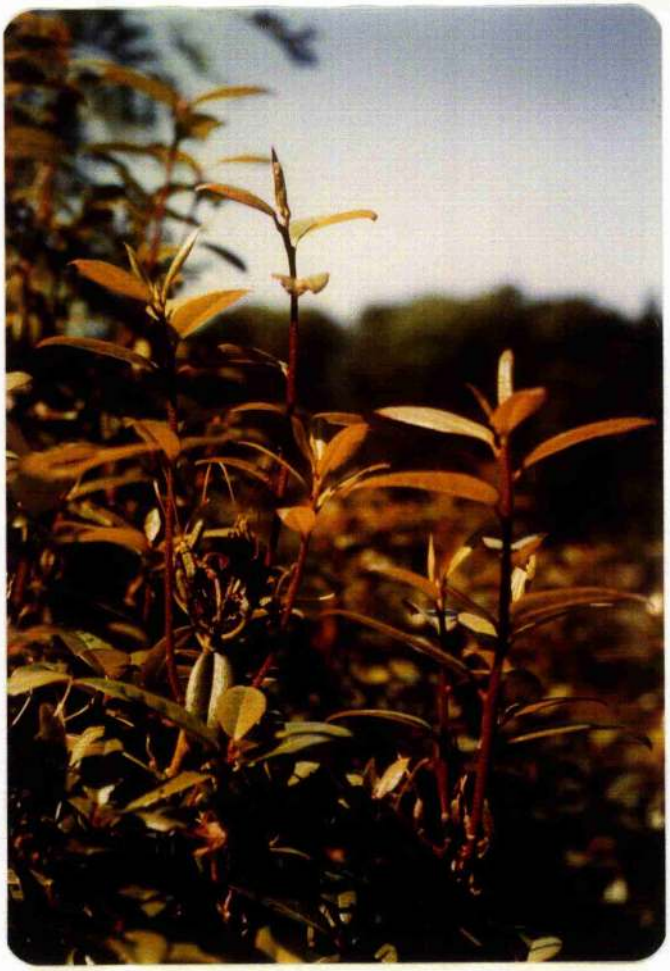


Plate 4.

Young shoots of <u>Rhododendron concinnum</u>

III. CAULOGENESIS

3.1 CYTOKININ ACTIVITY IN SHOOT FORMATION

This section tests the hypothesis that a high cytokinin / auxin ratio promotes shoot formation, in species of Rosaceae and Ericaceae. The effect of cytokinin concentration is examined to determine whether cytokinin concentration controls shoot formation.

Other workers have compared the activity of different cytokinins in the promotion of callus growth using the tobacco callus bioassay (Skoog et al., 1967; Schmitz et al., 1972) and in the promotion of cotyledon growth using the radish cotyledon bioassay (Letham, 1972). Different cytokinins have been used to induce shoot formation in different species, for example, N_6-benzyladenine in Malus (Lane, 1978), N_6-isopentenyl adenine in Rhododendron, kinetin in Gloxinia (Murashige, 1974) and zeatin in Nicotiana (Yamada et al, 1972).. However, few studies have compared cytokinins in the same species with respect to their activity in promoting the formation of shoots. In the current study, the activity of two cytokinins in promotion of shoot morphogenesis is studied in relation to cytokinin concentration and rate of shoot formation.

Although the main study is concerned with the regenerative capacity of excised shoots, shoot formation was also studied in whole plants to determine whether the initiation of adventitious shoots is limited to excised plant parts.

4.11 SHOOT FORMATION IN VITRO

Method

Cytokinins at the following concentrations were incorporated in the nutrient medium :-

BA : 0, 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0 mg l^{-1}

2iP : 0, 5.0, 10.0, 15.0 or 20.0 mg l^{-1} .

The species listed below were tested.

Rosaceae:- *Chaenomeles japonica*, *Cotoneaster dammeri*, *Crataegus brachyacantha*, *Crataegus* 'Toba', *Potentilla* 'Coronation Triumph', *Potentilla* 'Sutter's Gold', *Prunus cerasifera*, *Prunus tomentosa*, *Pyracantha coccinea*, *Spiraea* 'Froebelii'.

Ericaceae:- *Arctostaphylos media*, *Arctostaphylos uva-ursi*, *Erica carnea*, *Gaultheria hispidula*, *Kalmia angustifolia* (pink form), *Rhododendron arboreum*, *R. chamae-thomsonii*, *R.* 'Chikor',

R. 'Chinsayii', R. dauricum, R. fastigiatum, R. forrestii, R. keiskei, R. leucaspis, R. lutescens, R. 'P.J.M. Victor', R. racemosum, R. 'Vuyk's rosy red', R. williamsianum, Vaccinium vitis-idaea.

Cultures were incubated in light (16 hour photoperiod). Shoot number and length were recorded at the end of a four week incubation period. A four week period was chosen because preliminary experiments showed that most shoots were formed within four weeks and were long enough at the end of the four week period to record with the naked eye. Shoot length was recorded in 5 mm increments thus:-1-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35 mm.

Results

The addition of BA to the medium at all concentrations tested resulted in death of explants of Gaultheria hispidula, Rhododendron 'chinsayii' and Rhododendron dauricum. BA concentrations greater than 0.1 mg l^{-1} resulted in death of explants of Erica carnea, R. fastigiatum and R. P.J.M. Victor. No toxic effects due to 2 iP were observed.

Shoot number

Figures 1 to 24 show fitted polynomials for shoot

number plotted against BA concentration. Statistics of fit are included with each figure. Equations for curves are of the form :-

$y=b+b_1 \log_e x+b_2 \log_e x^2+b_3 \log_e x^3+b_4 \log_e x^4$

The probability of non-fit is

<.001 for every Rosaceous plant model

<.001 for Arctostaphylos

<.05 for Kalmia

<.001 to N.S. for Rhododendron

<.001 for Vaccinium.

Figures 25 to 54 show fitted polynomials for shoot number plotted against 2iP concentration. Equations are of the form :-

$y=b+b_1 x+b_2 x^2+b_3 x^3+b_4 x^4$

i.e. quartic equations. The probability is <.001 for every model.

These models were used to determine optimal shoot numbers and optimal cytokinin concentrations. These are detailed in Tables 2 to 5. In Rosaceae, the optimal concentration of BA for maximal shoot formation ranged from 0.4 to 2.3 mg l^{-1} whereas in Ericaceae, the optimal BA concentration ranged between 0.5 and 6.2 mg l^{-1}. Optimal concentration of 2ip was higher (4.3 to 17.2 mg l^{-1} in Rosaceae; 10.3 to 20.0 mg l^{-1} in

Ericaceae). Maximal calculated shoot number on medium containing BA was 5.34 to 26.40 in Rosaceae and 0.61 to 16.49 in Ericaceae; and on medium containing 2ip was 4.02 to 31.27 for Rosaceae and 2.13 to 11.78 in Ericaceae. These can be summarized as follows.

	mean shoot no.	mean conc.
BA Rosaceae	14.54	1.22
BA Ericaceae	2.76	2.96
2iP Rosaceae	10.25	12.53
2iP Ericaceae	5.27	15.03

The form of the curves was different for BA and for 2iP - different equations were necessary for plotting of the data. The spread of effective concentrations for shoot formation was great for all species for 2iP and for most species for BA. A few species, both Potentilla cultivars, Spiraea and Vaccinium, had a marked peak with a narrow concentration range.

An analysis of variance was conducted to show the main significant effects. These are detailed in Tables 6 to 9. This analysis showed that for both BA and 2iP, concentration had a significant effect on shoot number

($p<.001$), species and cultivar had a significant effect ($p<.001$) and that there was a significant interaction between concentration and species ($p<.001$).

Mean shoot number for BA was compared with mean shoot number for 2iP and was found to be significantly different for both Ericaceae and Rosaceae ($p<.001$)(Tables 10 and 11). In Rosaceae, BA gave consistently more shoots than 2iP, while in Ericaceae, 2iP gave consistently more shoots than BA.

Shoot length

An obvious decrease in shoot length was recorded with increasing BA concentration (Figures 55 to 81). However, change in shoot length with change in 2iP concentration approximated a normal distribution (Figures 82 to 117).

A comparison of shoot length between BA treatments with the maximal number of shoots and 2iP treatments with the maximal number of shoots demonstrated that shoot length was significantly greater in 2iP treatments than in BA treatments in all species tested ($p<.001$) (Tables 12 and 13).

Explant growth

A linear correlation between BA and 2iP concentration and explant growth was demonstrated

(Tables 14 to 17). Explant length decreased with increasing cytokinin concentration.

A comparison was made between explant growth on BA medium and explant growth on 2iP medium at the concentrations which produced most shoots (Tables 18 and 19). Explants grew significantly more on 2iP medium than on BA medium ($p<.001$) in all species.

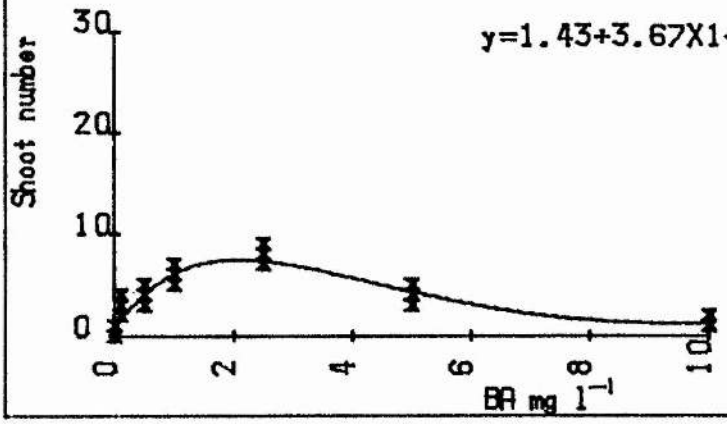
Origin of shoots

New shoots formed were either axillary in origin or adventitious (derived from a part of the stem other than the leaf axils). Adventitious shoots were formed directly from the stem (not from basal callus). Species which formed adventitious shoots also produced axillary shoots. Types of shoots produced for each species are given in Tables 20 and 21. Species of Rosaceae formed both adventitious and axillary shoots on medium containing BA but only four cultivars formed adventitious shoots on 2iP medium. All Ericaceous species (with 3 exceptions) formed axillary shoots only on both BA and 2iP media.

Figures 1 to 24.

Shoot number after a four week incubation period on medium containing BA.

Fig 1. Chaenomeles japonica.



$y=1.43+3.67X1+11.33X2-11.73X3+2.64X4$

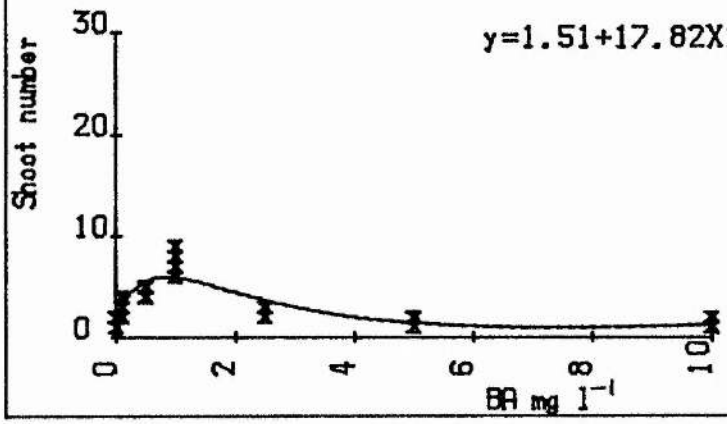
R2= .8294

r= .9107

(p=<.001)

S.E.= 1.1

Fig 2. Cotoneaster dammeri.



$y=1.51+17.82X1-21.91X2+8.51X3-1.05X4$

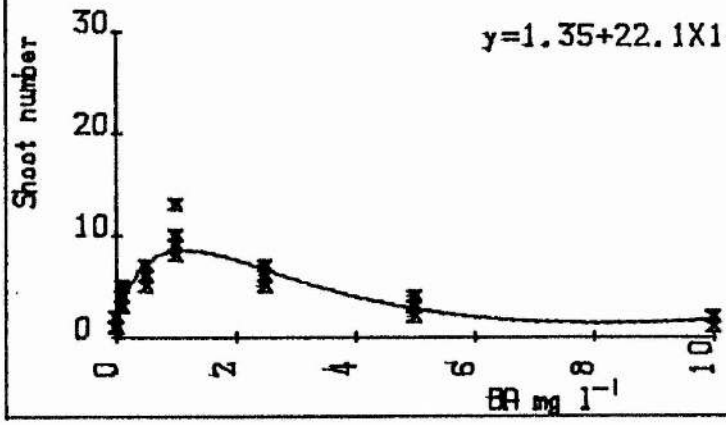
R2= .7572

r= .8702

(p=<.001)

S.E.= 1.147

Fig 3. Crataegus brachyacantha.



$y=1.35+22.1X1-20.03X2+4.49X3+.02X4$

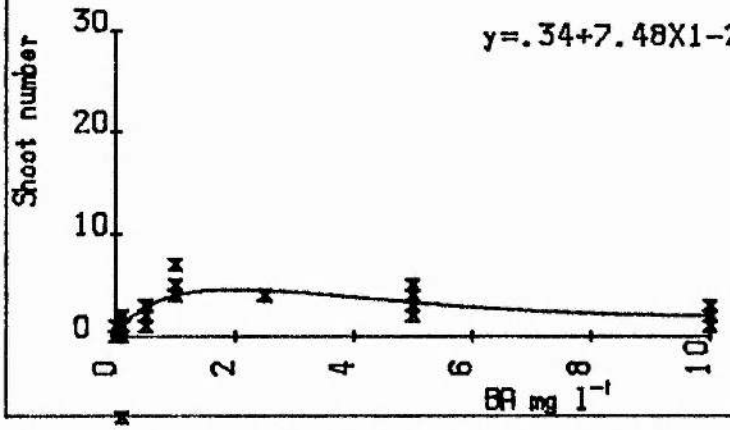
R2= .8209

r= .906

(p=<.001)

S.E.= 1.38

Fig 4. Crataegus 'Toba'.



y=.34+7.48X1-2.19X2-1.6X3+.55X4

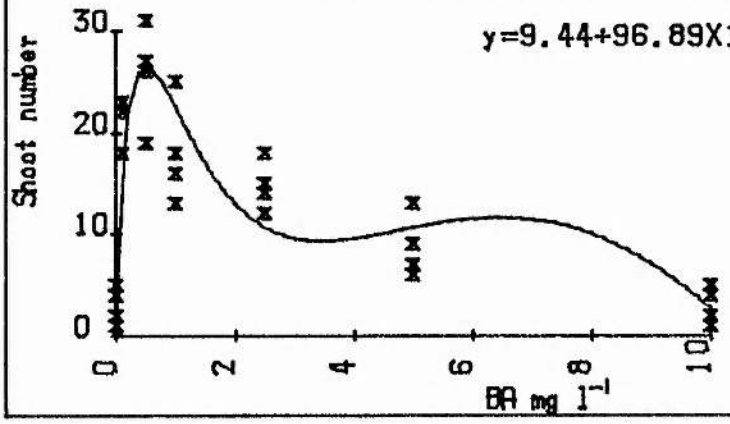
R2= .693

r= .8324

(p=<.001)

S.E.= 1.05

Fig 5. Potentilla 'Coronation Triumph'.



y=9.44+96.89X1-174.21X2+102.59X3-19.73X4

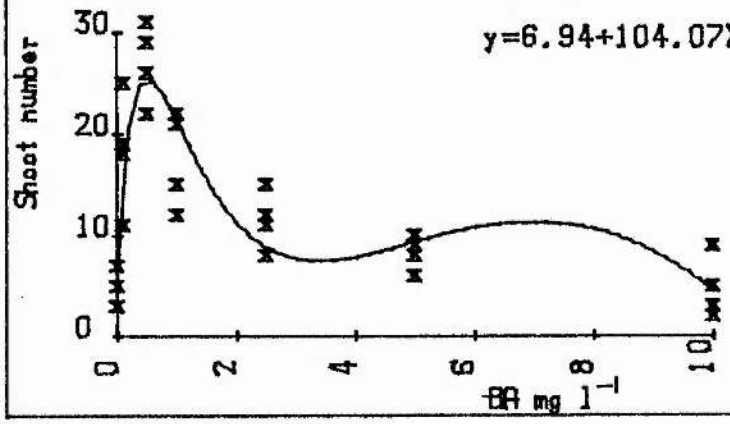
R2= .5704

r= .7553

(p=<.001)

S.E.= 7.309

Fig 6. Potentilla 'Sutter's Gold'.



y=6.94+104.07X1-185.08X2+107.97X3-20.47X4

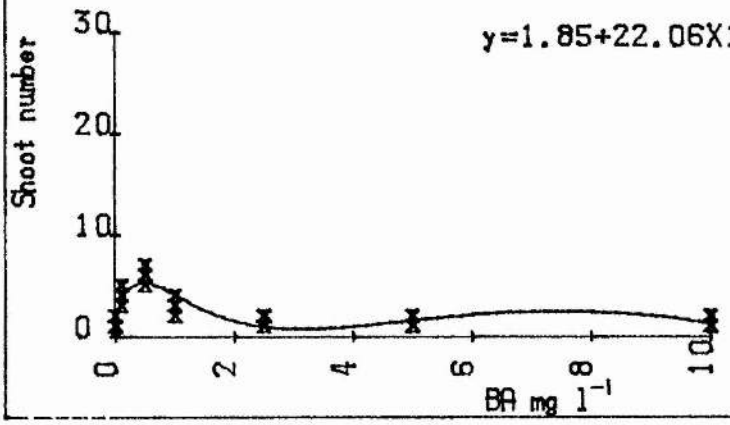
R2= .7637

r= .8739

(p=<.001)

S.E.= 4.443

Fig 7. Prunus cerasifera.



y=1.85+22.06X1-42.99X2+26.04X3-5.01X4

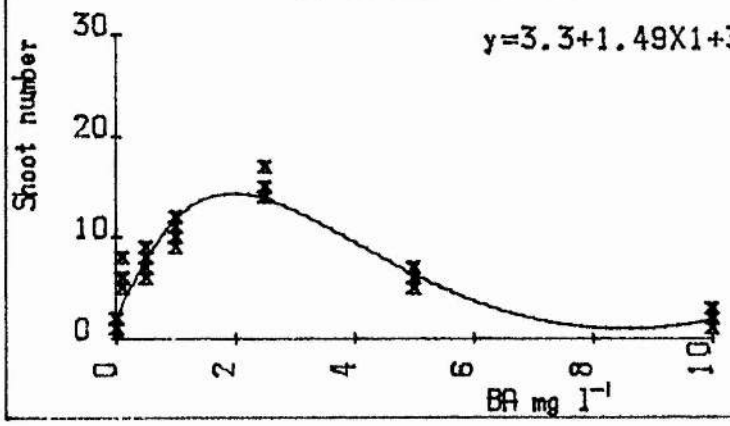
R2= .7271

r= .8527

(p=<.001)

S.E.= 1.047

Fig 8. Prunus tomentosa.



y=3.3+1.49X1+34.5X2-32.35X3+7.33X4

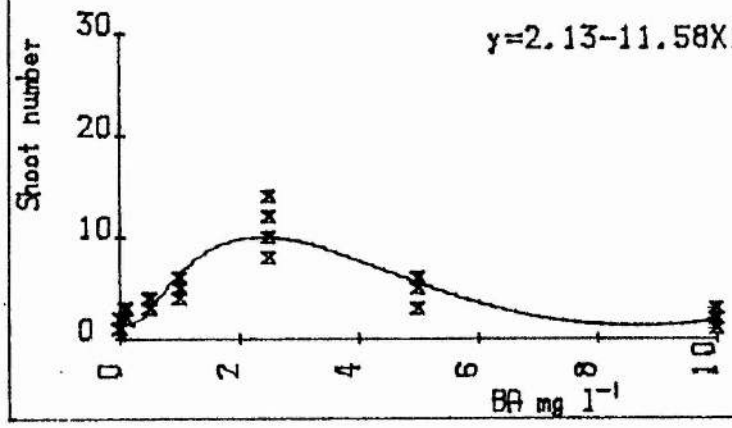
R2= .8591

r= .9268

(p=<.001)

S.E.= 1.855

Fig 9. Pyracantha coccinea.



y=2.13-11.58X1+45.81X2-34.06X3+7.06X4

R2= .8108

r= .9004

(p=<.001)

S.E.= 1.544

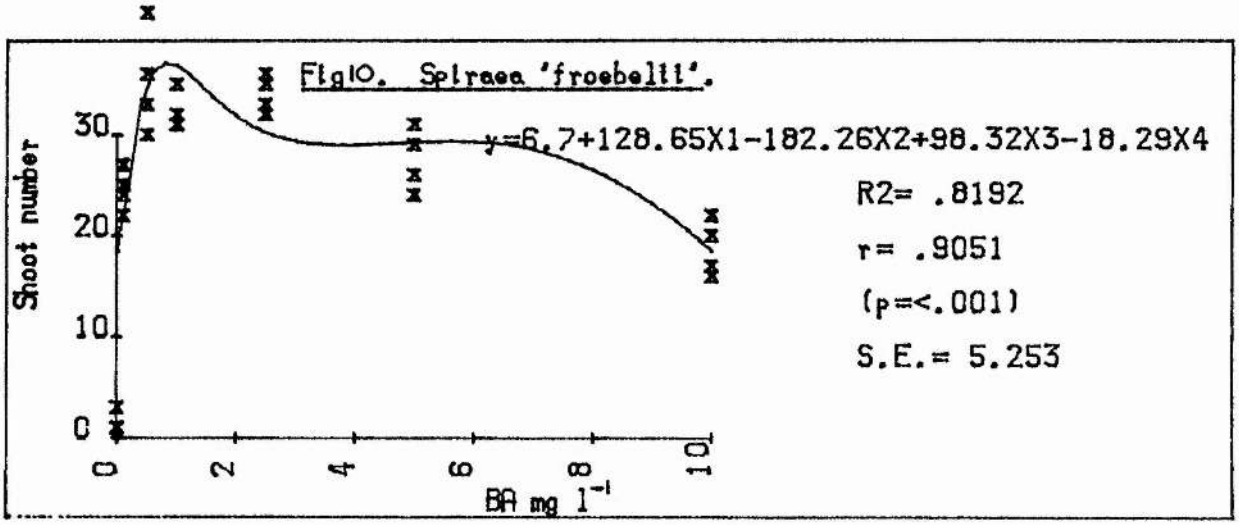


Fig10. Spiraea 'froebelii'.
y=6.7+128.65X1-182.26X2+98.32X3-18.29X4
R2= .8192
r= .9051
(p=<.001)
S.E.= 5.253
Shoot number
30
20
10
0
0
2
4
6
8
10
BA mg l⁻¹

Fig 11. Arctostaphylos media.

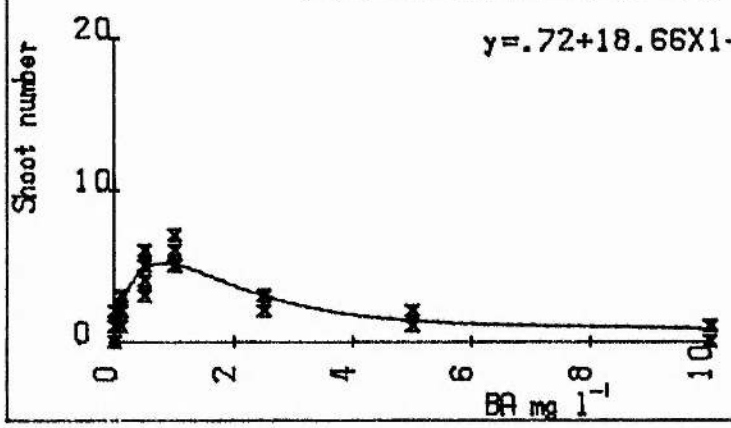


Fig 12. Arctostaphylos uva-ursi.

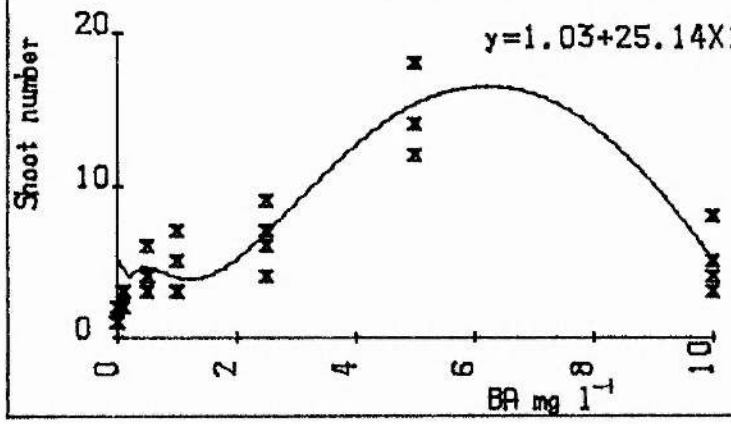


Fig 13. Kalmia angustifolia.

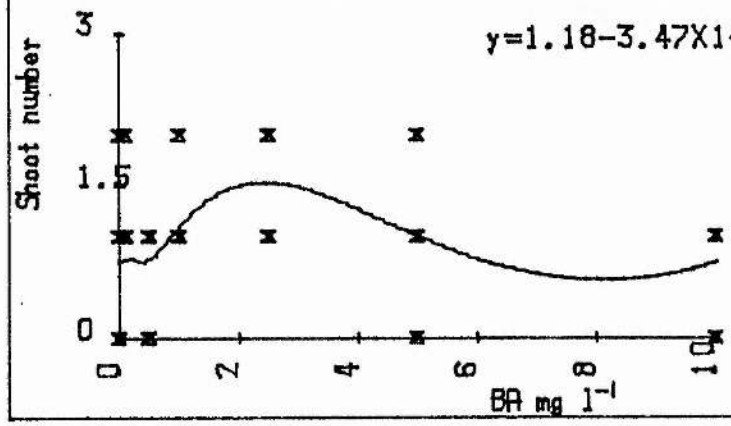


Fig 14. Rhododendron arboreum.

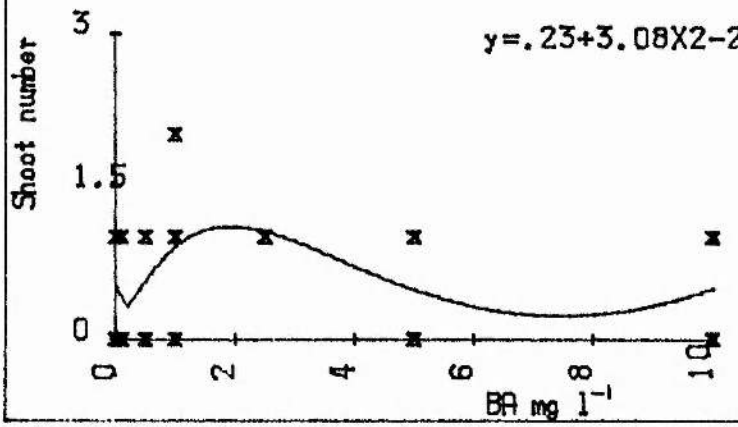


Fig 15. Rhododendron chamae-thomsonii.

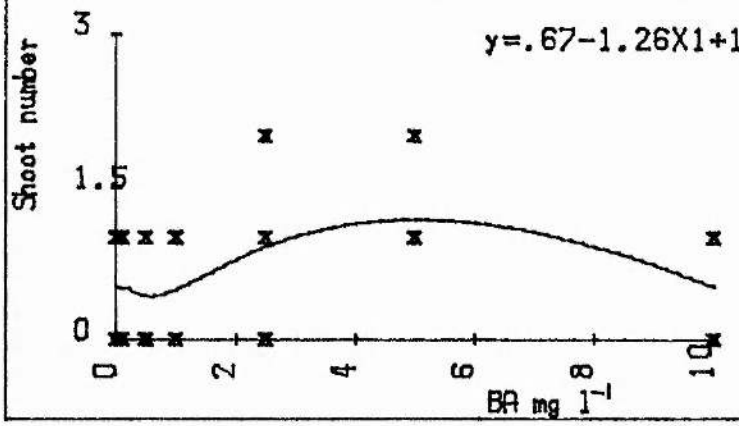


Fig 16. Rhododendron 'chikor.'

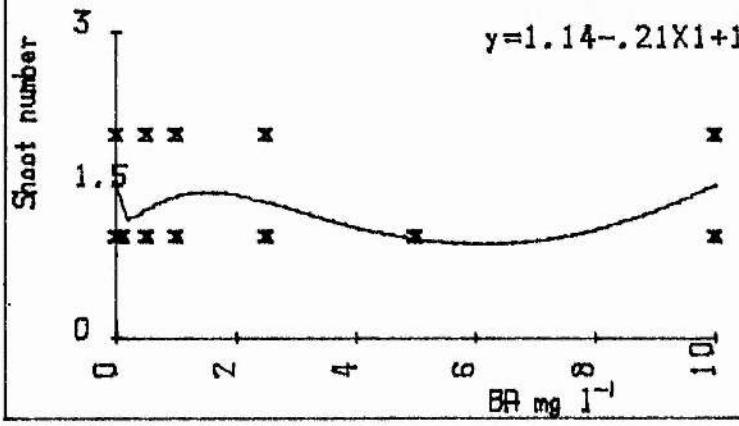
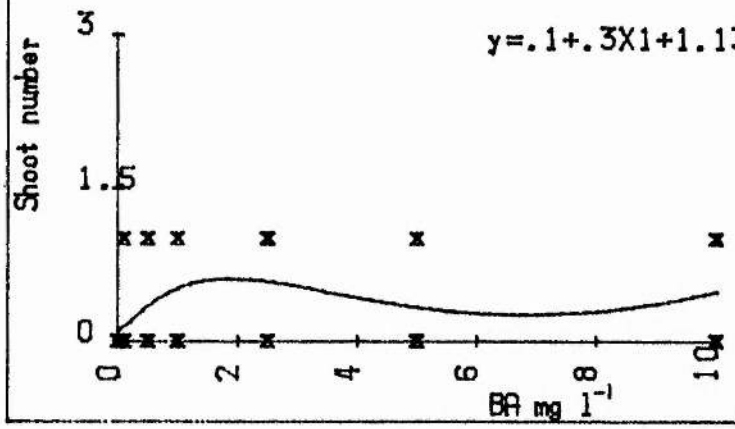


Fig 17. Rhododendron forrestii.



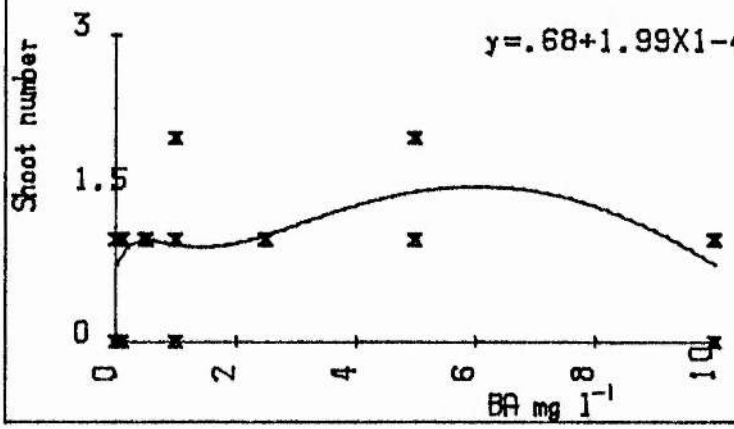
y=.1+.3X1+1.13X2-1.27X3+.31X4

R2= .1259

r= .3548

S.E.= .494

Fig 18. Rhododendron keiskei.



y=.68+1.99X1-4.18X2+3.16X3-.74X4

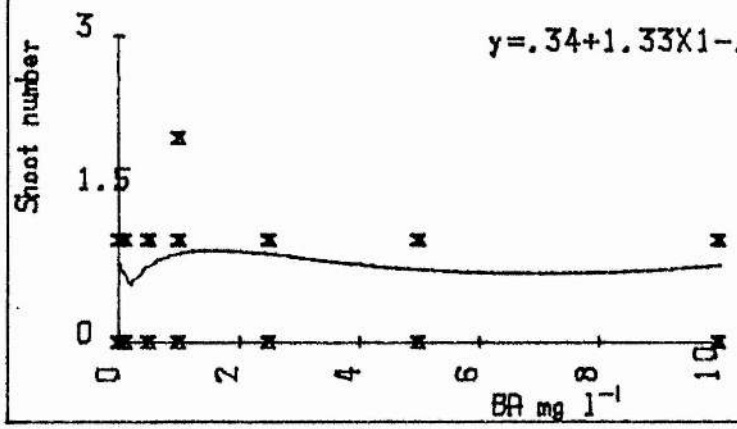
R2= .236

r= .4858

(p=<.05)

S.E.= .48

Fig 19. Rhododendron leucaspis.



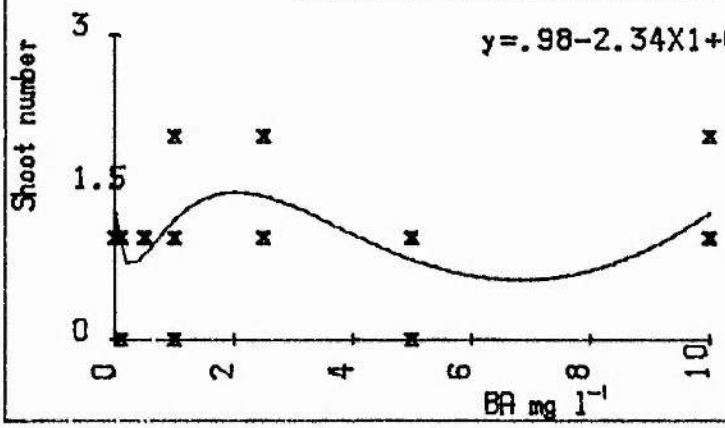
y=.34+1.33X1-.88X2+.05X3+.04X4

R2= .1184

r= .3441

S.E.= .557

Fig 20. Rhododendron lutescens.



y=.98-2.34X1+6.76X2-5.12X3+1.13X4

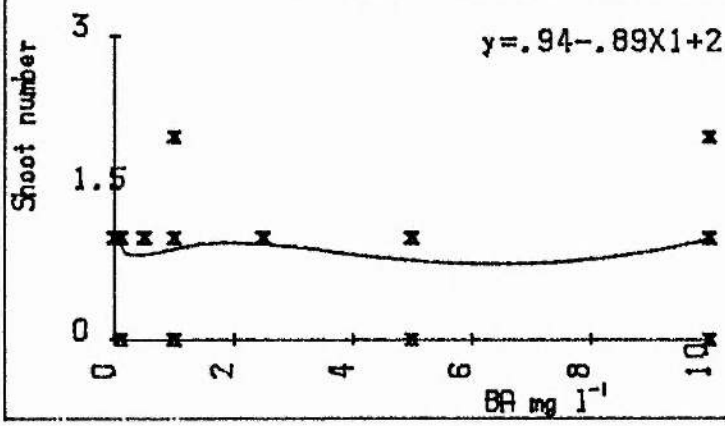
R2= .206

r= .4538

(p=<.05)

S.E.= .49

Fig 21. Rhododendron racemosum.



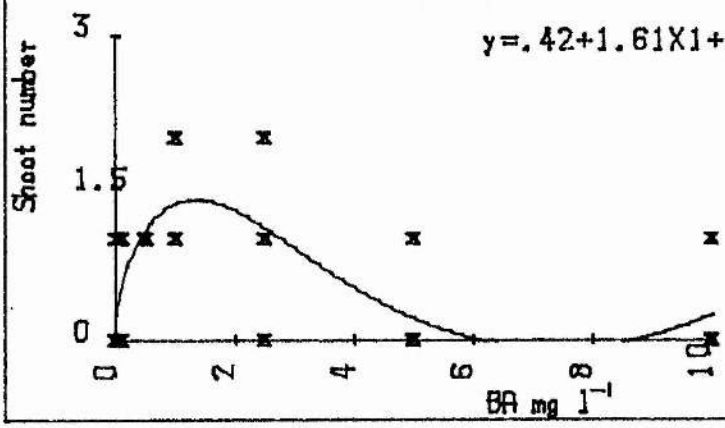
y=.94-.89X1+2.06X2-1.51X3+.33X4

R2= .0202

r= .1422

S.E.= .533

Fig 22. Rhododendron Vuyk's.



y=.42+1.61X1+.65X2-1.92X3+.56X4

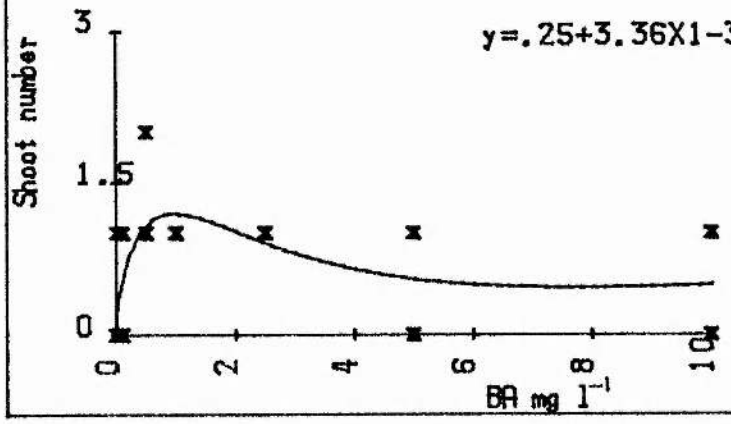
R2= .4274

r= .6537

(p=<.001)

S.E.= .54

Fig 23. Rhododendron williamsianum.



y=.25+3.36X1-3.82X2+1.44X3-.18X4

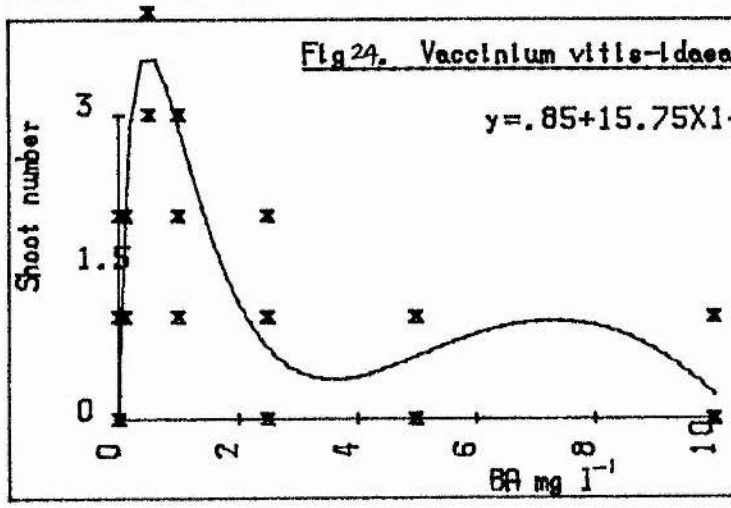
R2= .3748

r= .6122

(p=<.001)

S.E.= .457

Fig 24. Vaccinium vitis-idaea.



y=.85+15.75X1-28.31X2+16.25X3-3.02X4

R2= .687

r= .8288

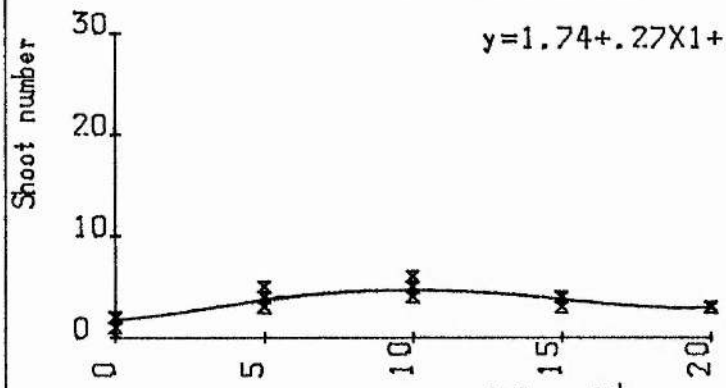
(p=<.001)

S.E.= .894

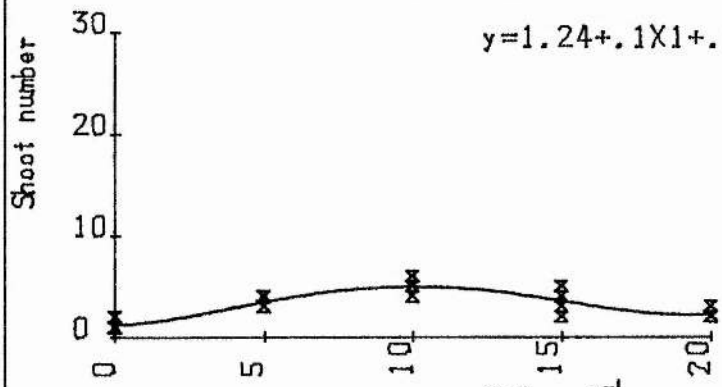
Figures 25 to 54.

Shoot number after a four week incubation period on medium containing 2iP.

Flg25. Cotoneaster dammeri



Flg26. Crataegus 'brachyacantha'.



Flg27. Crataegus 'Toba'.

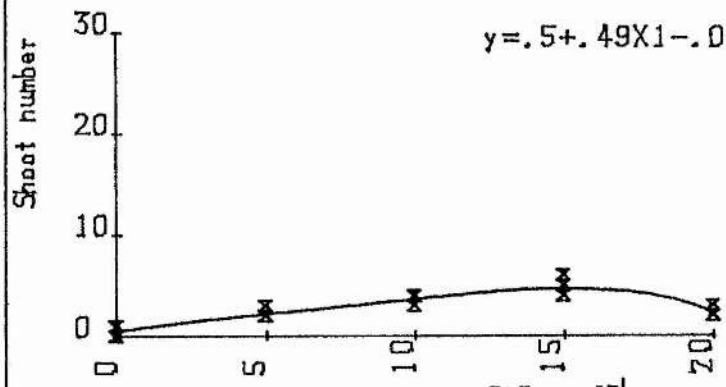


Fig28. Chaenomeles 'japonica'.

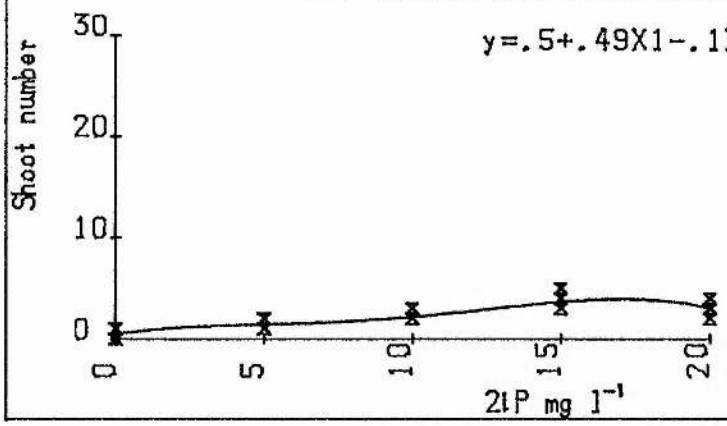


Fig29. Pyracantha coccinea.

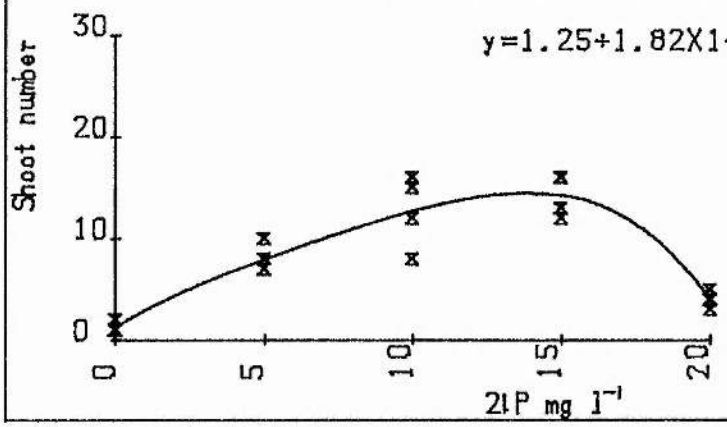


Fig30. Prunus tomentosa.

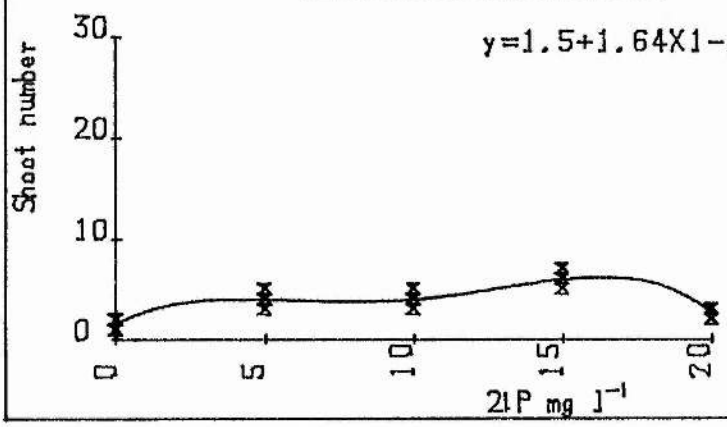
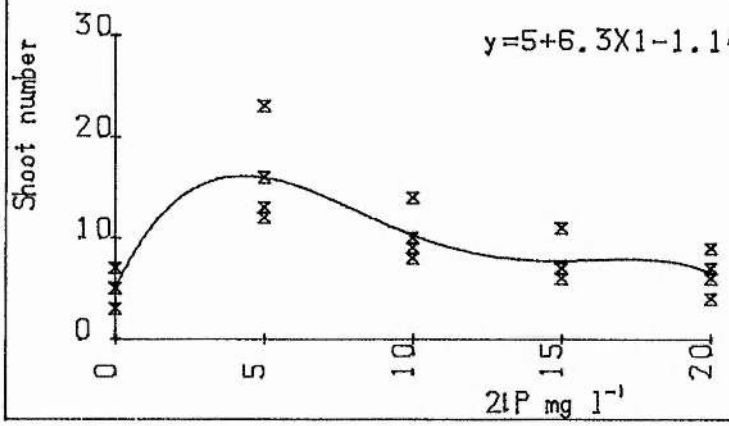


Fig31. Potentilla 'Sutter's Gold.'



y=5+6.3X1-1.14X2+.07X3-.01X4

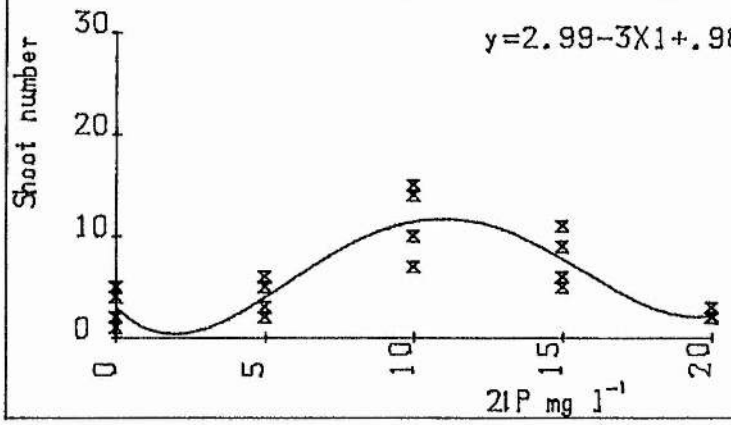
R2= .6949

r= .8336

(p=<.001)

S.E.= 2.949

Fig32. Potentilla 'Coronation triumph.'



y=2.99-3X1+.98X2-.08X3

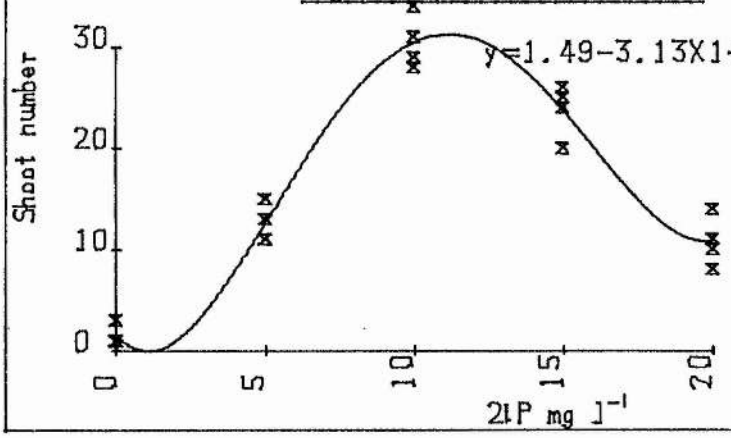
R2= .7393

r= .8598

(p=<.001)

S.E.= 2.373

Fig33. Spiraea 'Froebelii.'



y=1.49-3.13X1+1.69X2-.15X3

R2= .9654

r= .9825

(p=<.001)

S.E.= 2.228

Fig 34. Prunus cerasifera.

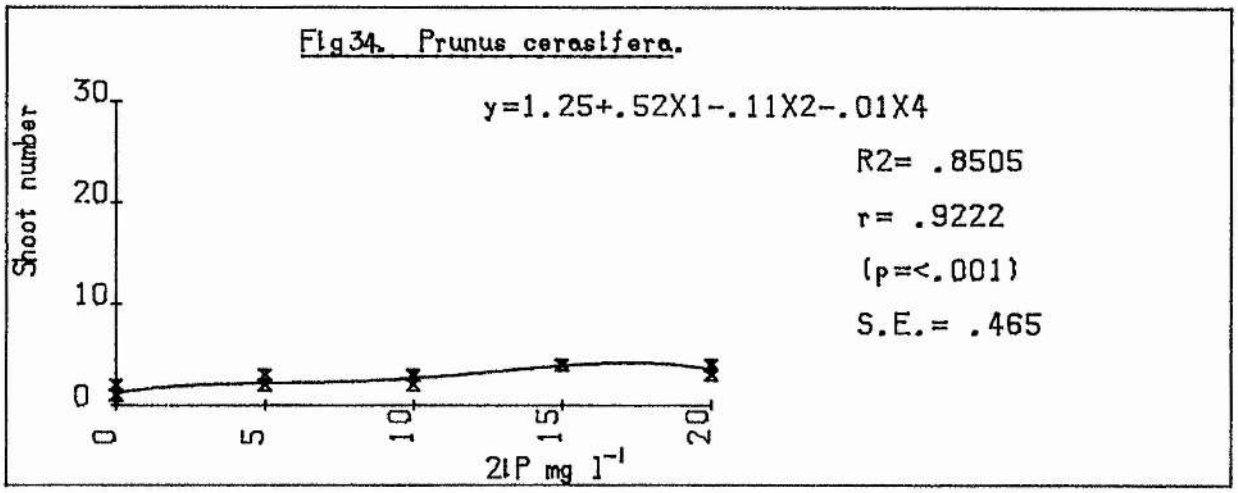
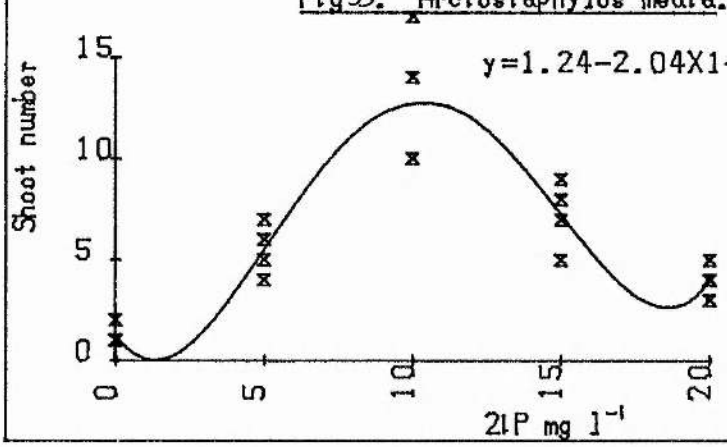


Fig35. Arctostaphylos media.



y=1.24-2.04X1+.93X2-.09X3

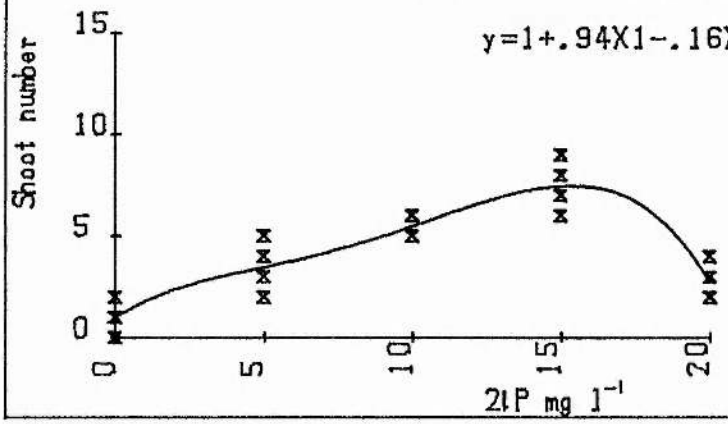
R2= .8521

r= .923

(p=<.001)

S.E.= 1.848

Fig36. Arctostaphylos uva-ursi.



y=1+.94X1-.16X2+.01X3-.01X4

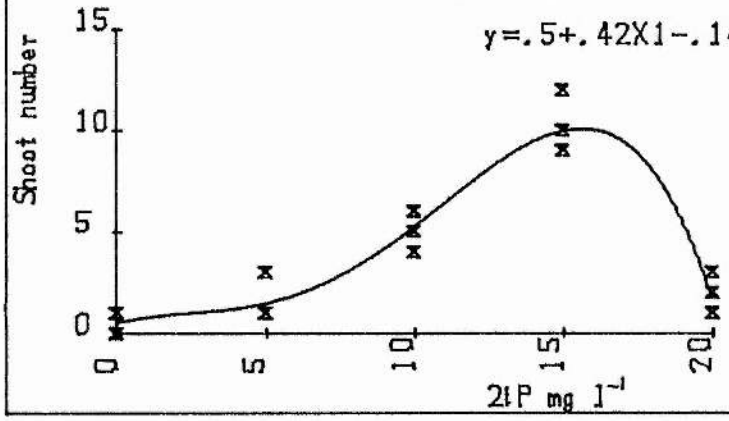
R2= .8653

r= .9302

(p=<.001)

S.E.= 1.024

Fig37. Erica carnea.



y=.5+.42X1-.14X2+.02X3-.01X4

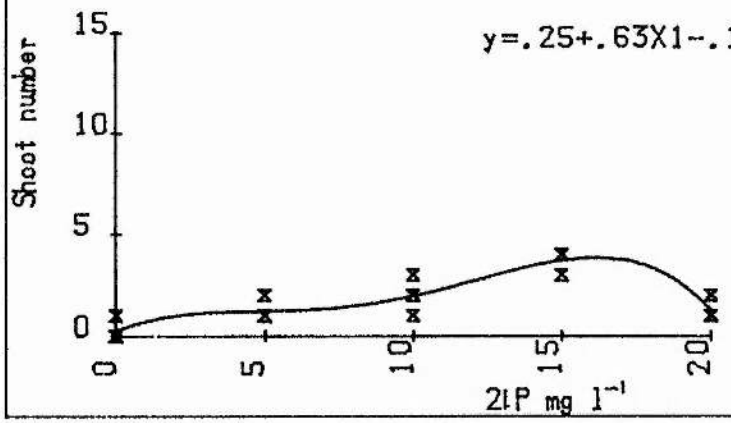
R2= .9402

r= .9696

(p=<.001)

S.E.= 1.016

Fig38. Gaultheria hispidula.



y=.25+.63X1-.15X2+.01X3-.01X4

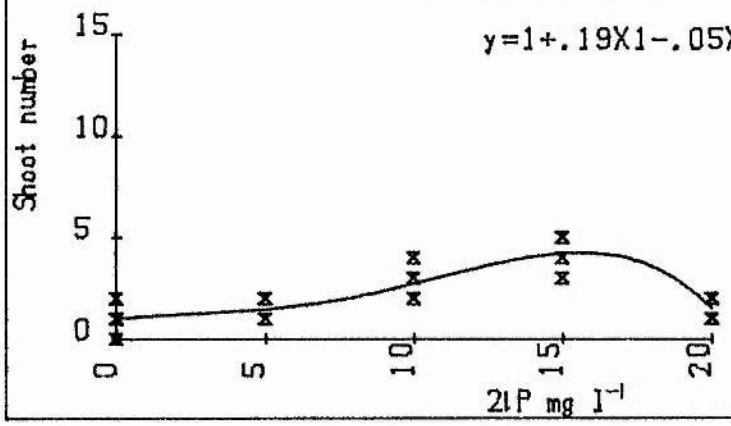
R2= .8447

r= .919

(p=<.001)

S.E.= .577

Fig39. Kalmia angustifolia.



y=1+.19X1-.05X2-.01X4

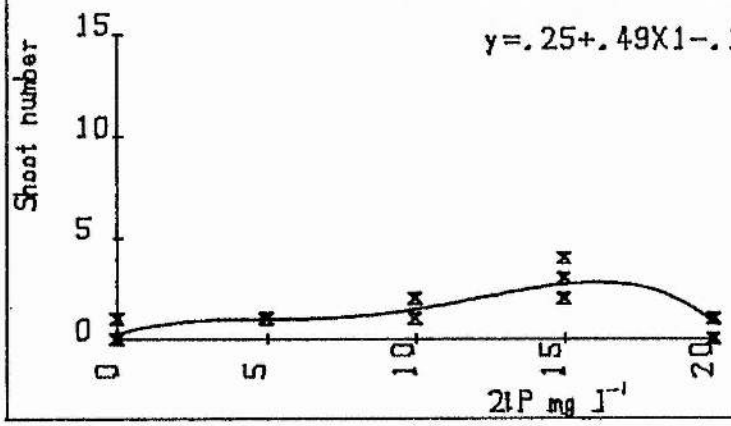
R2= .7446

r= .8629

(p=<.001)

S.E.= .795

Fig40. Rhododendron arboreum.



y=.25+.49X1-.12X2+.01X3-.01X4

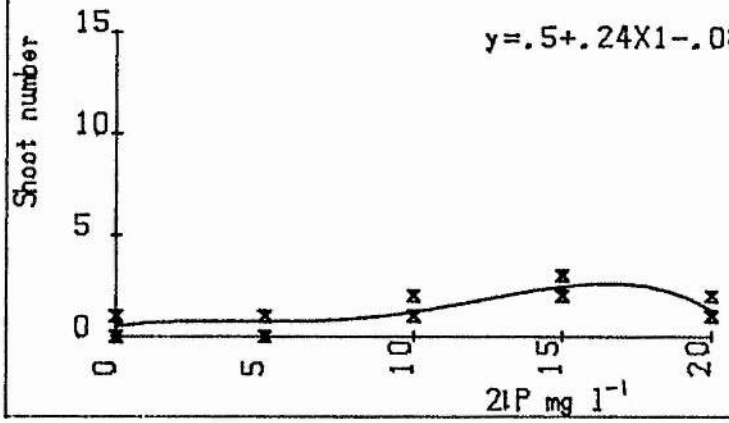
R2= .7341

r= .8568

(p=<.001)

S.E.= .591

Fig41. Rhododendron chamae-thomsonii.



y=.5+.24X1-.08X2-.01X4

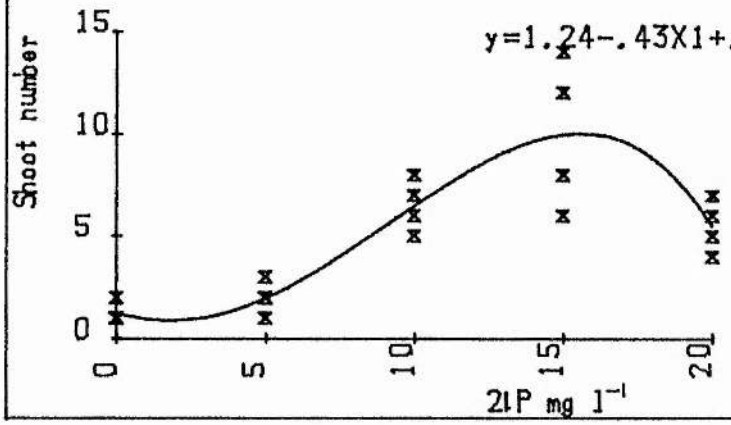
R2= .6909

r= .8312

(p=<.001)

S.E.= .532

Fig42. Rhododendron chikor.



y=1.24-.43X1+.12X2-.01X3-.01X4

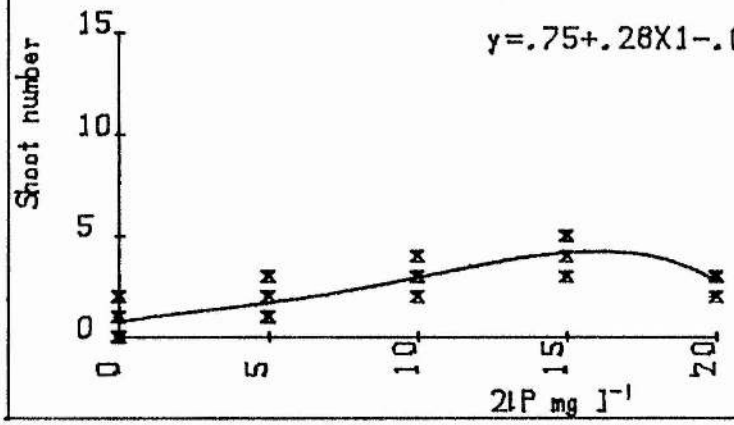
R2= .793

r= .8905

(p=<.001)

S.E.= 1.875

Fig43. Rhododendron chinsayii.



y=.75+.28X1-.04X2-.01X4

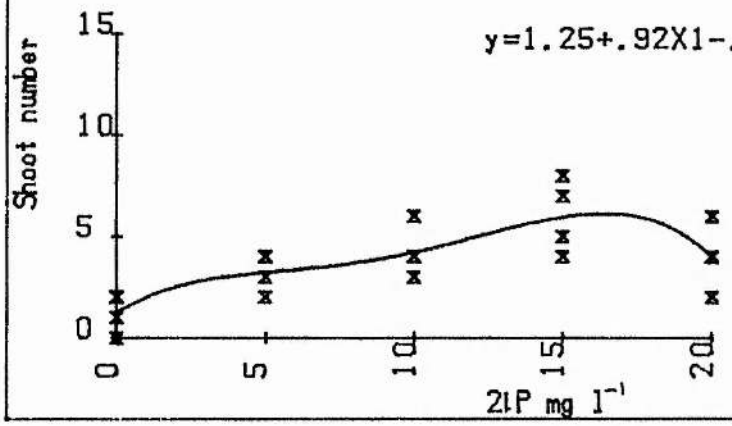
R2= .7179

r= .8473

(p=<.001)

S.E.= .856

Fig44. Rhododendron dauricum.



y=1.25+.92X1-.17X2+.01X3-.01X4

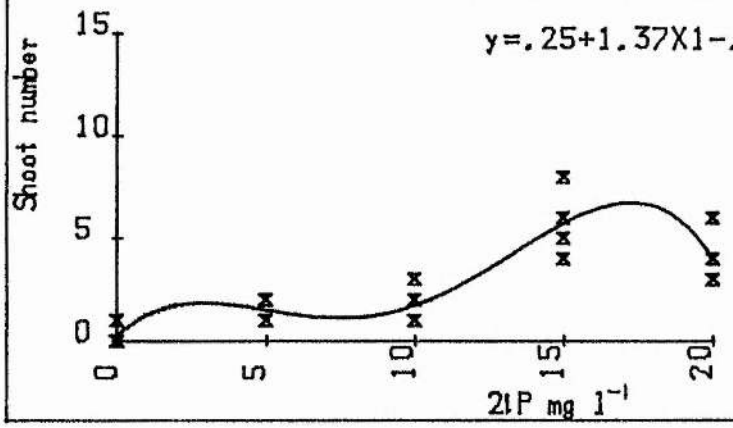
R2= .627

r= .7918

(p=<.001)

S.E.= 1.372

Fig45. Rhododendron fastigiatum.



y=.25+1.37X1-.38X2+.03X3-.01X4

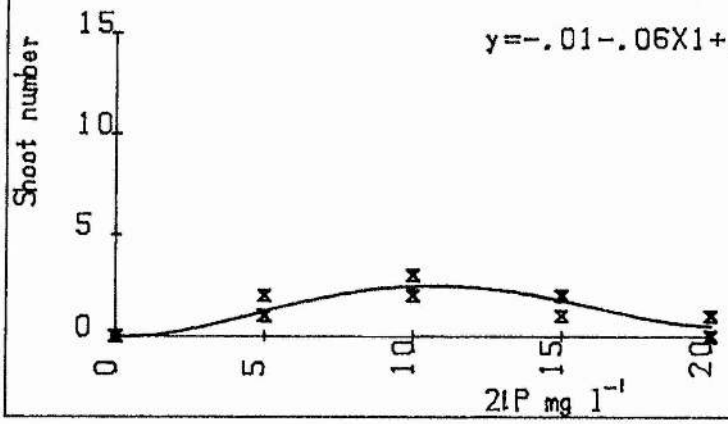
R2= .8006

r= .8947

(p=<.001)

S.E.= 1.132

Fig46. Rhododendron forrestii.



y=-.01-.06X1+.1X2-.01X3

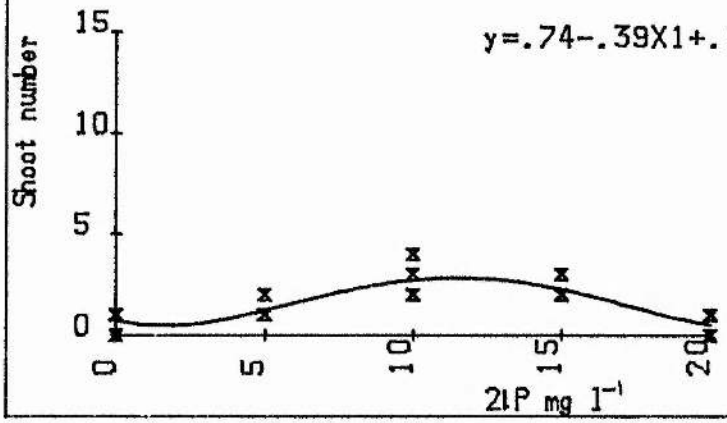
R2= .8177

r= .9042

(p=<.001)

S.E.= .483

Fig47. Rhododendron keiskei.



y=.74-.39X1+.14X2-.02X3

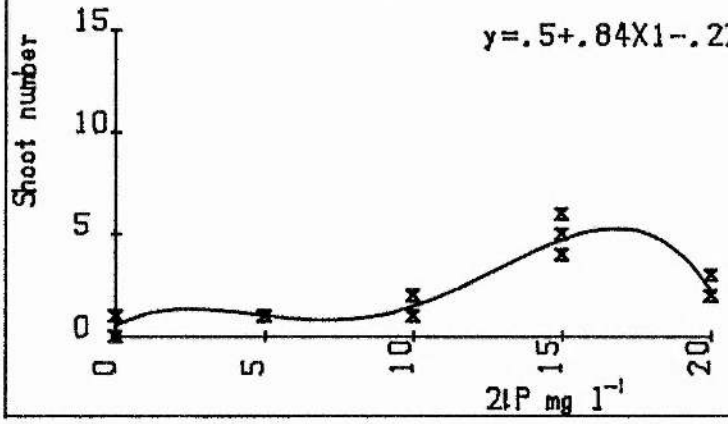
R2= .7142

r= .8451

(p=<.001)

S.E.= .632

Fig48. Rhododendron leucaspis.



y=.5+.84X1-.27X2+.02X3-.01X4

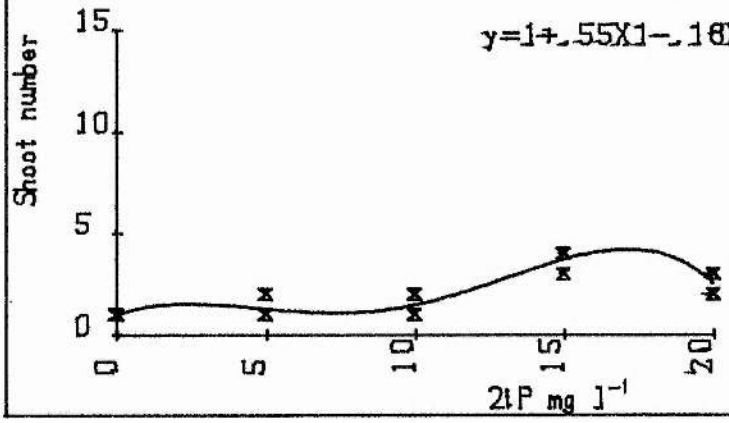
R2= .8899

r= .9433

(p=<.001)

S.E.= .605

Fig49. Rhododendron lutescens.



y=1+.55X1-.18X2+.01X3-.01X4

R2= .8541

r= .9242

(p=<.001)

S.E.= .483

Fig 50. Rhododendron PJM'Victor.

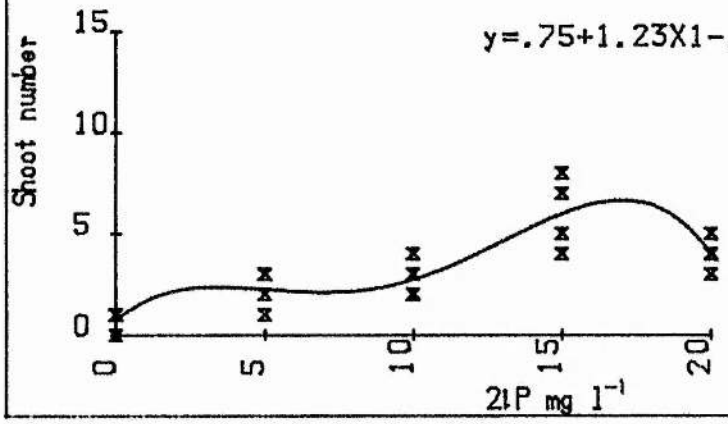


Fig 51. Rhododendron racemosum.

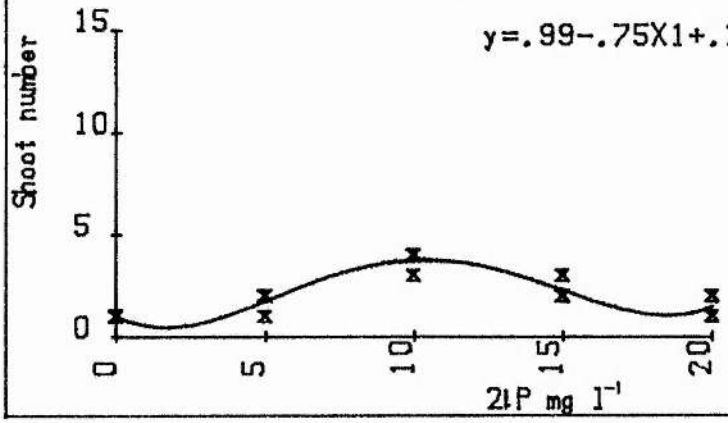


Fig 52. Rhododendron Vuyk's.

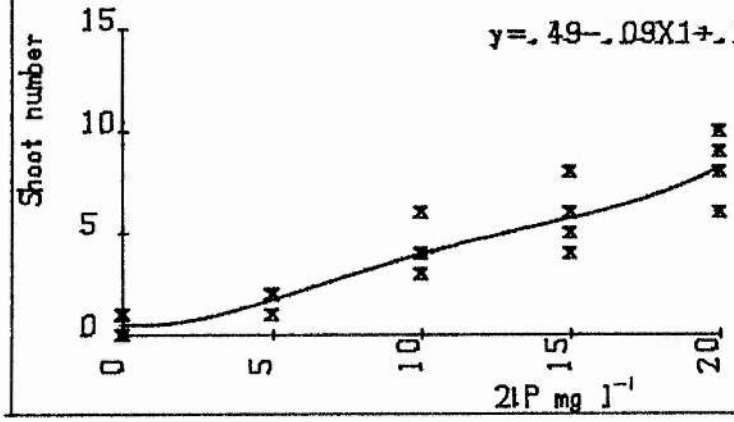


Fig 53. Rhododendron williamsianum.

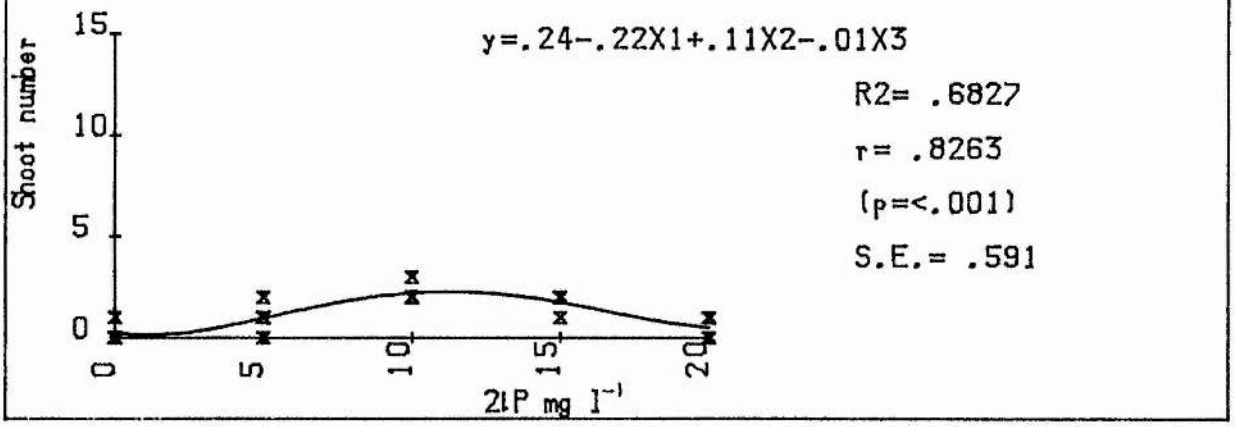


Fig 54. Vaccinium vitis-adaea.

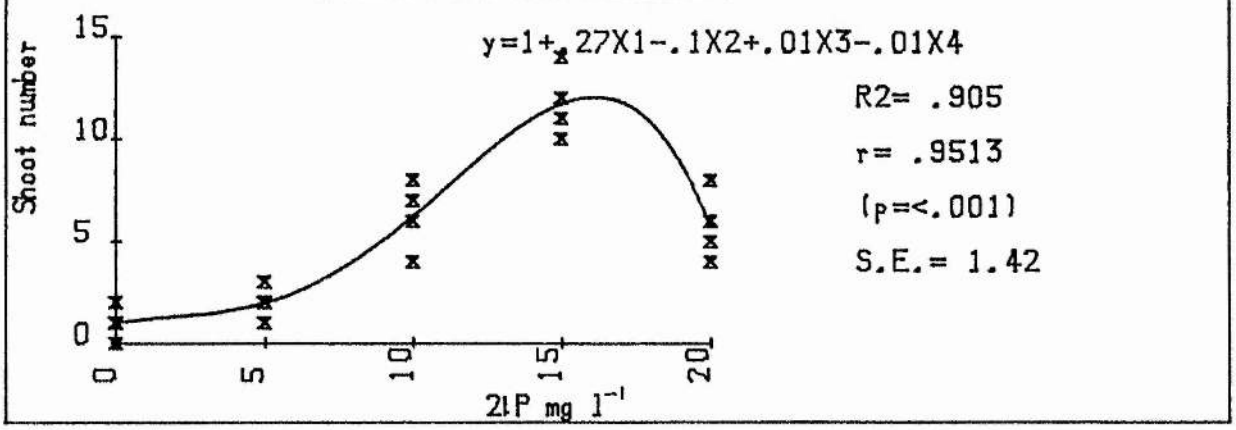


Table 2.

Maximal shoot number calculated from polynomial curves (on medium containing BA): Rosaceae.

Species	Optimal Concentration BA mg l^{-1}.	Maximal Shoot Number
Chaenomeles japonica	2.0	7.46
Cotoneaster dammeri	0.8	6.03
Crataegus brachyacantha	1.1	8.57
Crataegus 'Toba'	1.9	4.63
Potentilla 'Coronation Triumph'	0.5	26.40
Potentilla 'Sutter's Gold'	0.5	25.36
Prunus cerasifera	0.4	5.34
Prunus tomentosa	1.9	14.39
Pyracantha coccinea	2.3	10.04
Spiraea 'Froebelii'	0.8	37.14

Table 3.

Maximal shoot number calculated from polynomial curves (on medium containing BA): Ericaceae.

Species	Optimal Concentration BA mg l^{-1}.	Maximal Shoot Number
Arctostaphylos media	0.8	5.18
Arctostaphylos uva-ursi	6.2	16.49
Kalmia angustifolia	2.4	1.53
Rhododendron arboreum	1.9	1.11
Rhododendron chamae-thomsonii	5.0	1.18
Rhododendron 'chikor'	1.0	1.49
Rhododendron forrestii	1.8	0.61
Rhododendron keiskei	6.0	1.52
Rhododendron leucaspis	1.5	0.90
Rhododendron lutescens	2.0	1.45
Rhododendron racemosum	10	1.00
Rhododendron 'Vuyk's'	1.3	1.39
Rhododendron williamsiasnum	1.0	1.20
Vaccinium vitis-idaea	0.5	3.59

Table 4.

Maximal shoot number calculated from polynomial curves on medium containing 2iP : Rosaceae.

Species	Optimal Concentration 2iP mg l^{-1}.	Maximal Shoot Number
Chaenomeles japonica	17.0	4.02
Cotoneaster dammeri	9.8	4.75
Crataegus brachyacantha	9.9	5.00
Crataegus 'Toba'	15.0	4.75
Potentilla 'Coronation Triumph'	10.9	11.74
Potentilla 'Sutter's Gold'	4.3	15.92
Prunus cerasifera	17.2	4.28
Prunus tomentosa	16.2	6.20
Pyracantha coccinea	13.8	14.52
Spiraea 'Froebelii'	11.2	31.27

Table 5.

Maximal shoot number calculated from polynomial curves on medium containing 2iP: Ericaceae.

Species	Optimal Concentration 2iP mg l^{-1}.	Maximal Shoot Number
Arctostaphylos media	10.3	11.78
Arctostaphylos uva-ursi	15.3	6.51
Erica carnea	15.6	9.60
Gaultheria hispidula	16.1	3.62
Kalmia angustifolia	15.5	3.28
Rhododendron arboreum	16.0	2.58
Rhododendron chamae-thomsonii	16.4	2.13
Rhododendron 'Chikor'	15.5	8.79
Rhododendron chinsayii	15.9	3.55
Rhododendron dauricum	16.3	4.91
Rhododendron fastigiatum	17.2	6.51
Rhododendron forrestii	10.6	2.51
Rhododendron keiskei	11.4	2.09
Rhododendron leucaspis	16.8	4.80
Rhododendron lutescens	17.0	3.23
Rhododendron 'P.J.M.Victor'	17.0	5.92
Rhododendron racemosum	10.3	2.76
Rhododendron 'Vuyk's'	20.0	7.75
Rhododendron williamsianum	11.2	2.06
Vaccinium vitis-idaea	16.1	11.07

Table 6.

Analysis of variance for shoot number after BA treatment : Rosaceae.

SOURCE	S.S.	D.F.	M.S.	F	p
Concentration	3863.58	6	643.93	117.00	<.001
Variety	13273.95	9	1474.88	267.99	<.001
Interaction	4799.63	54	88.88	16.150	<.001
Error	1155.75	210	5.504		
Total	23092.91	279			

Table 7.

Analysis of variance for shoot number after BA treatment : Ericaceae.

SOURCE	S.S.	D.F.	M.S.	F	p
Concentration	61.526	6	10.254	18.134	<.001
Variety	678.472	13	52.190	92.294	<.001
Interaction	606.974	78	7.781	13.761	<.001
Error	166.250	294	.5655		
Total	1513.222	391			

Table 8.

Analysis of variance for shoot number after 2iP treatment : Rosaceae.

SOURCE	S.S.	D.F.	M.S.	F	p
Concentration	1308.870	4	327.218	125.853	<.001
Variety	3317.720	9	368.636	141.783	<.001
Interaction	1986.030	36	55.168	21.218	<.001
Error	390.000	150	2.600		
Total	7002.620	199			

Table 9.

Analysis of variance for shoot number after 2iP treatment : Ericaceae.

SOURCE	S.S.	D.F.	M.S.	F	p
Concentration	949.990	4	237.498	227.089	<.001
Variety	870.828	19	45.833	43.824	<.001
Interaction	766.510	76	10.0857	9.6441	<.001
Error	313.750	3001.0458			
Total	2901.078	399			

Table 10.

Comparison of mean shoot number (over all species) for best three treatments : Rosaceae (D.F.=39).

2iP	BA	t	p
8.75	12.15	7.56	<.001
7.93	11.42	7.78	<.001
5.73	10.83	11.33	<.001

Table 11.

Comparison of mean shoot number (over all species) for best three treatments : Ericaceae (D.F.=77).

2iP	BA	t	p
5.20	1.86	21.9	<.001
3.70	1.66	13.40	<.001
2.76	1.59	7.71	<.001

Figures 55 to 81.

Modal shoot length after four weeks on medium containing BA.

Fig 55. Chaenomeles japonica.

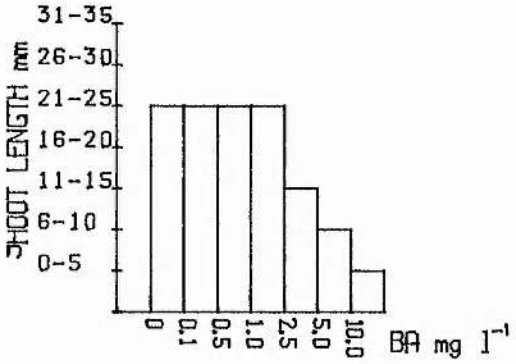


Fig 56. Cotoneaster dammeri.

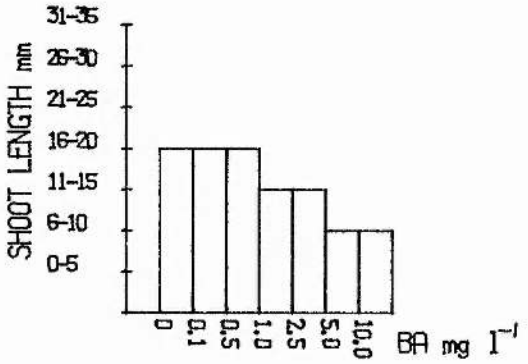


Fig 57. Crataegus brachyacantha.

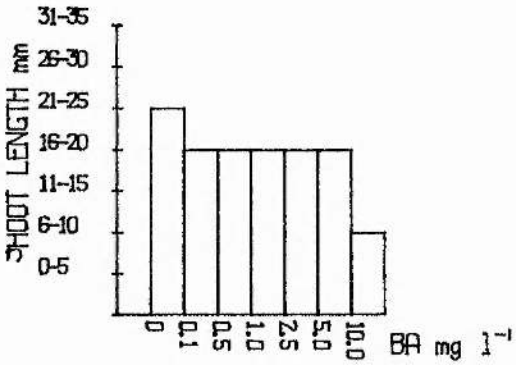


Fig 58. Crataegus 'Toba'.

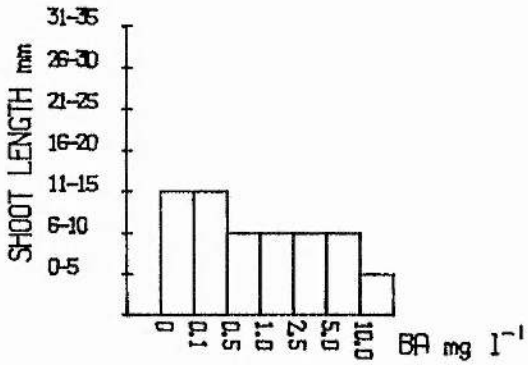


Fig 59. Potentilla 'Coronation Triumph'.

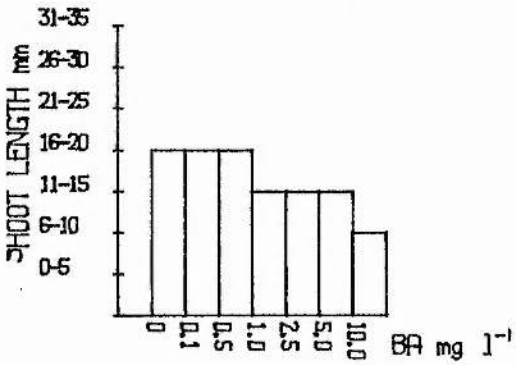


Fig 60. Potentilla 'Sutter's Gold'

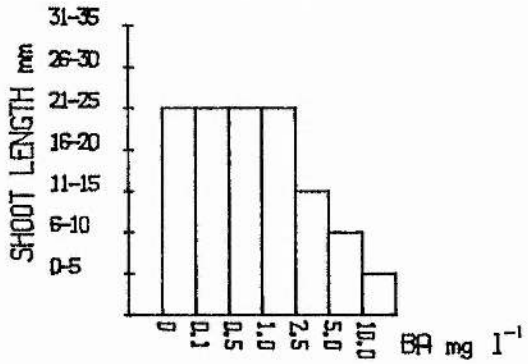


Fig 61 . Prunus ceraslfera.

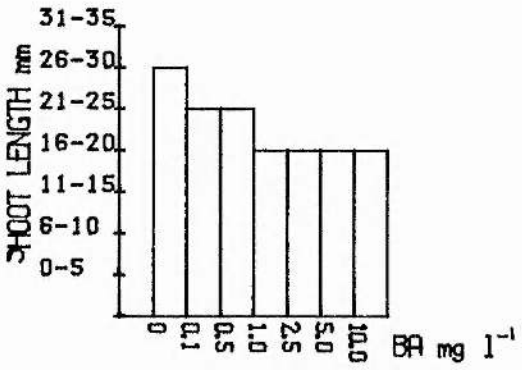


Fig 62 . Prunus tomentosa.

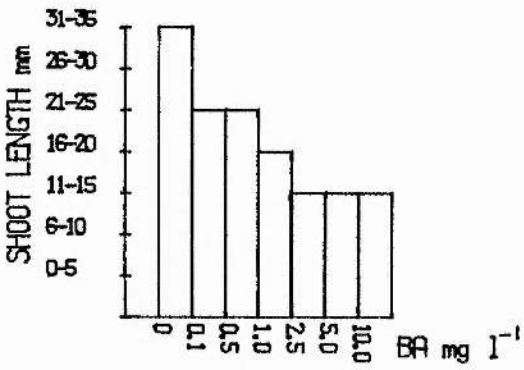


Fig 63 . Pyracantha coccinea.

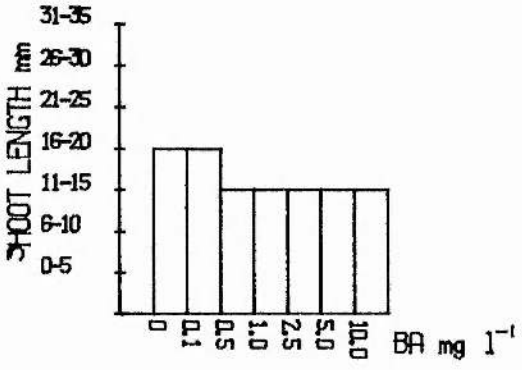


Fig 64 . Spiraea 'Froebelli'.

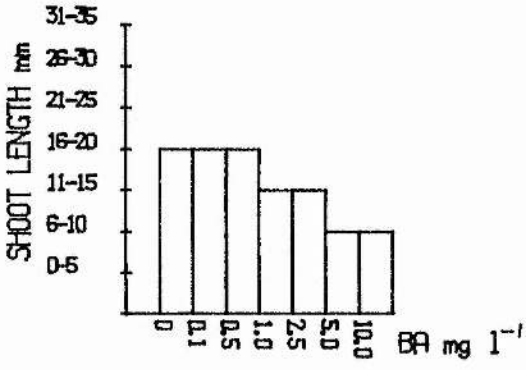


Fig 65. Arctostaphylos media.

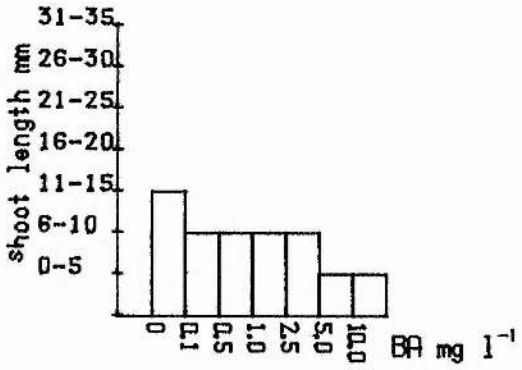


Fig 66. Arctostaphylos uva-ursi.

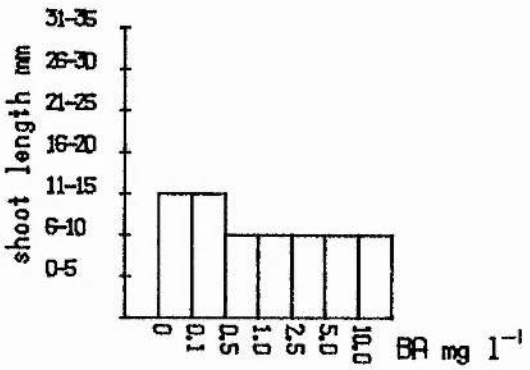


Fig 67. Erica carnea.

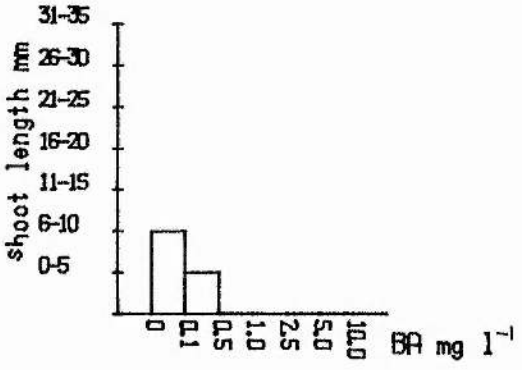


Fig 68. Kalmia angustifolia.

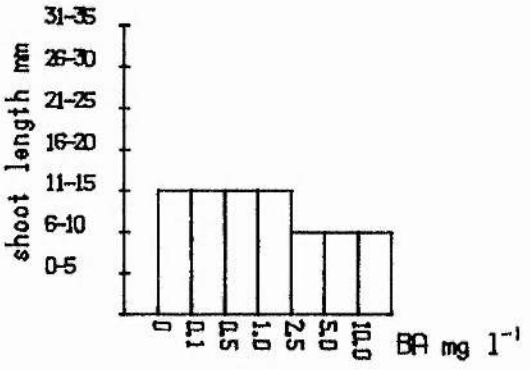


Fig 69. Rhododendron arboreum.

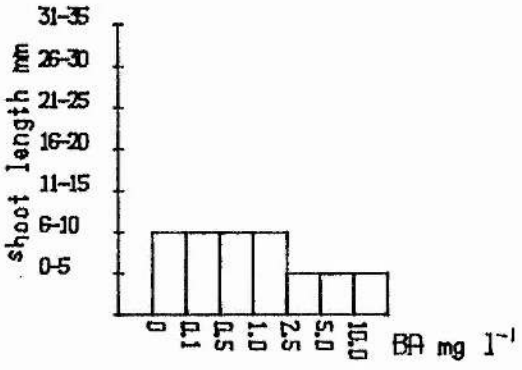


Fig 70. Rhododendron chamaethomsonii.

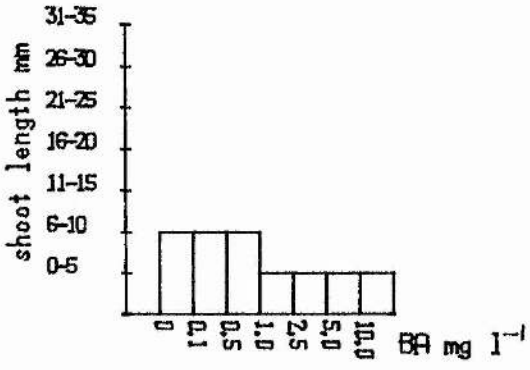


Fig 71 . Rhododendron chikor.

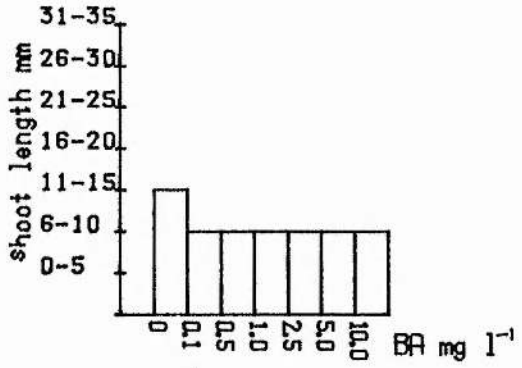


Fig 72 . Rhododendron fastigiatum.

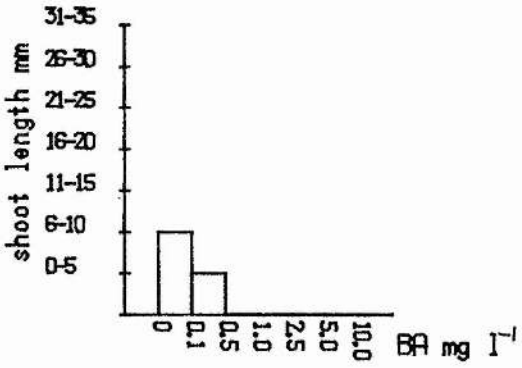


Fig 73 . Rhododendron forrestii.

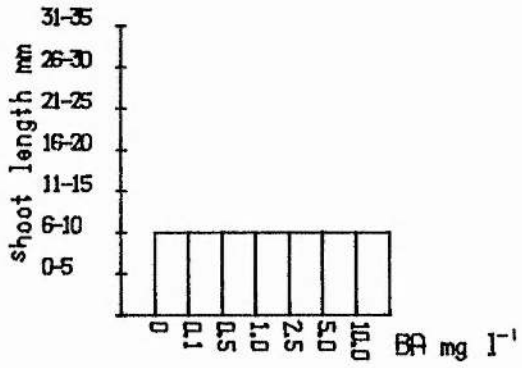


Fig 74 . Rhododendron keiskei.

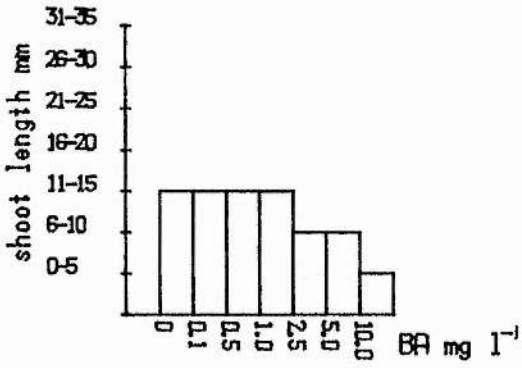


Fig 75 . Rhododendron leucaspis.

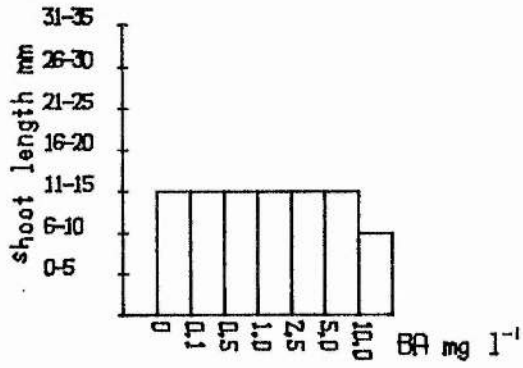


Fig 76 . Rhododendron lutescens.

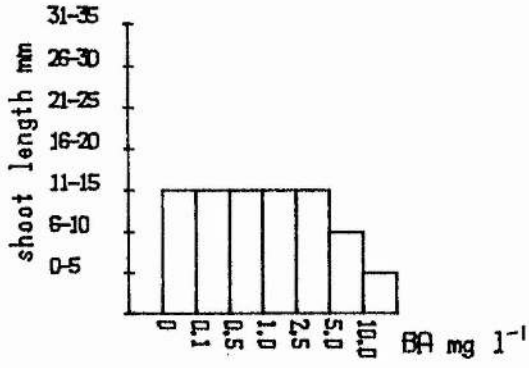


Fig 77. Rhododendron P.J.M' Victor.

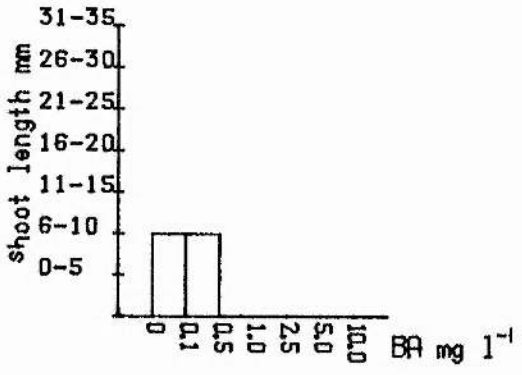


Fig 78. Rhododendron racemosum.

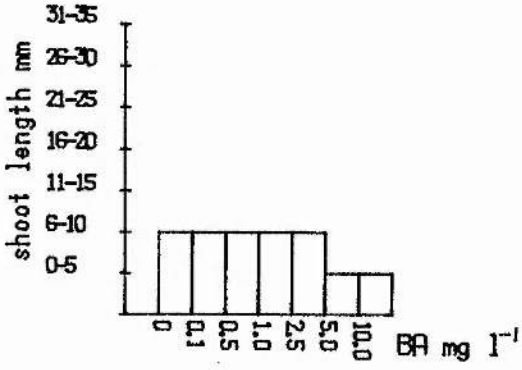


Fig 79. Rhododendron 'vuyks'.

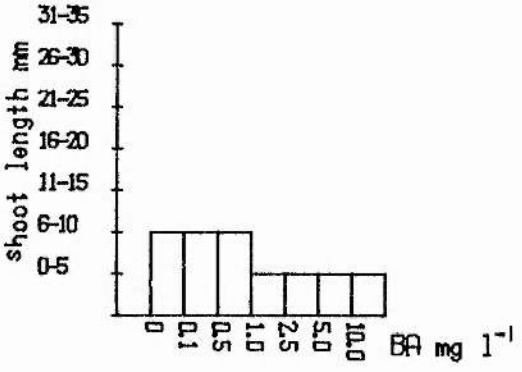


Fig 80. Rhododendron williamsianum.

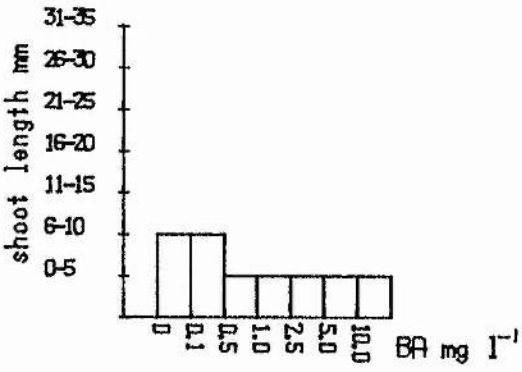
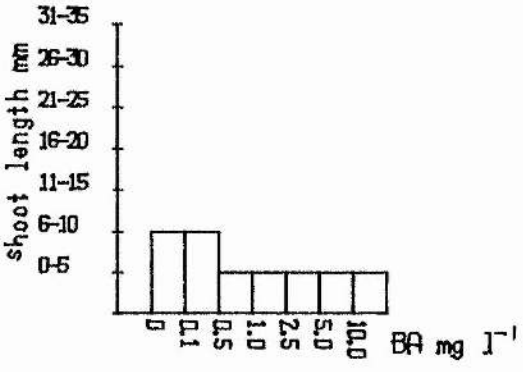


Fig 81. Vaccinium vitis-idaea.



Figures 82 to 117.

Modal shoot length after four weeks incubation on medium containing 2iP.

Fig 82. Chaenomeles japonica.

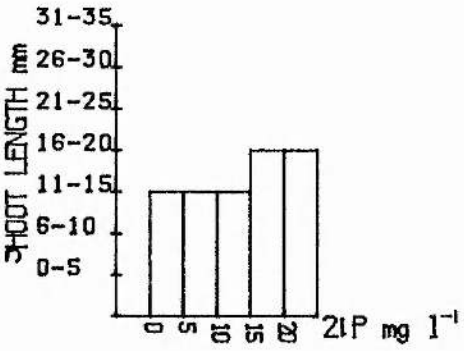


Fig 83. Cotoneaster dammeri.

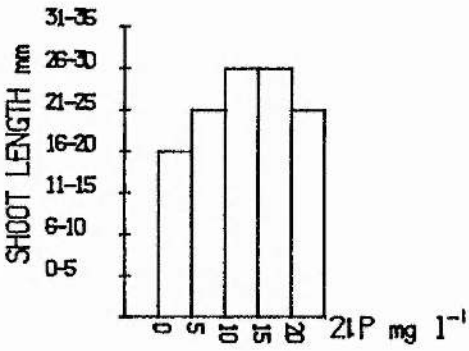


Fig 84. Crataegus brachyacantha.

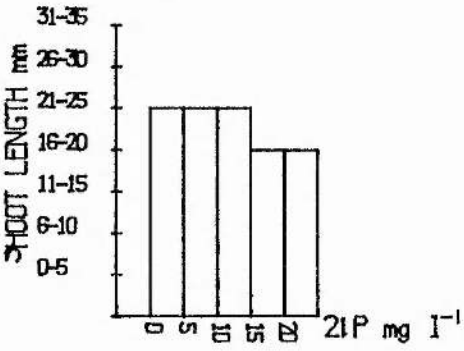


Fig 85. Crataegus 'Toba'.

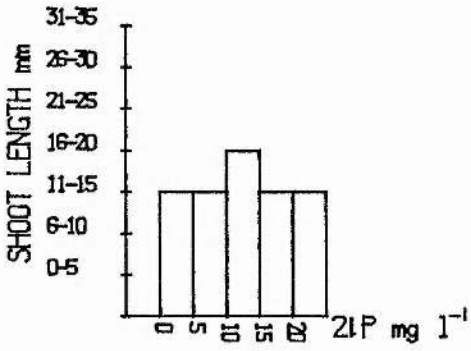


Fig 86. Potentilla 'Coronation Triumph'.

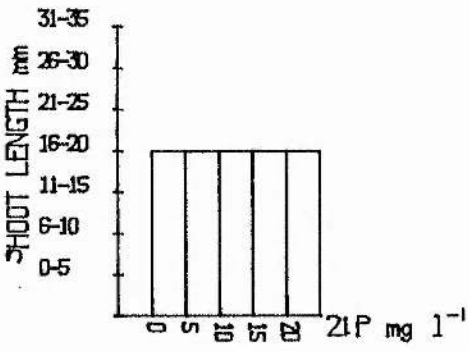


Fig 87. Potentilla 'Sutter's Gold'

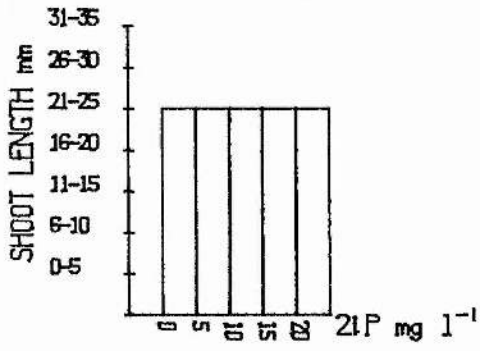


Fig 88 . Prunus cerasifera.

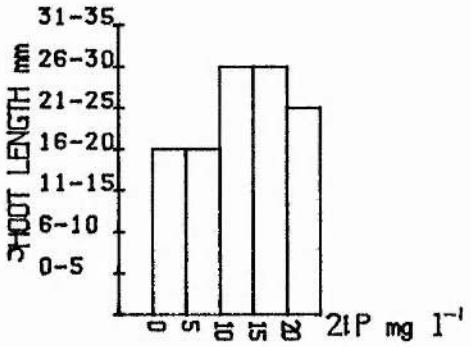


Fig 89 . Prunus tomentosa.

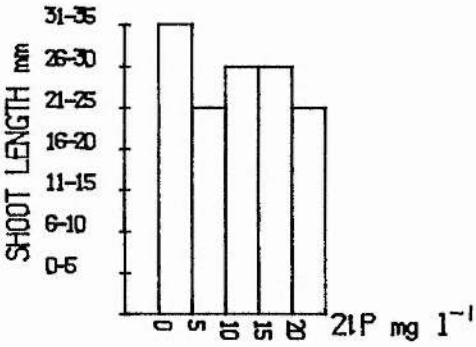


Fig 90 . Pyracantha coccinea.

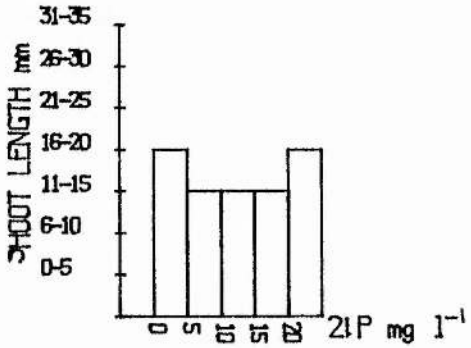


Fig 91 . Spiraea 'Froebelli'.

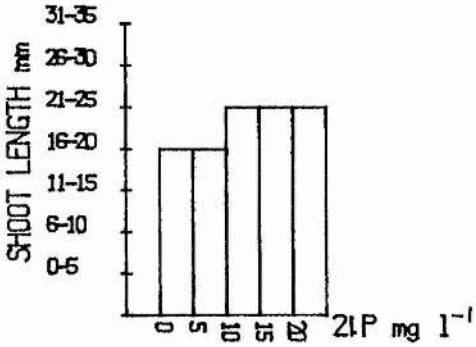


Fig 92 . Arctostaphylos media.

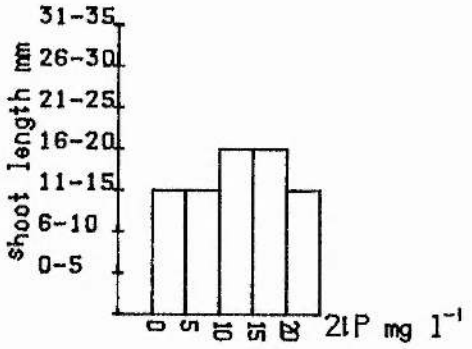


Fig 93 . Arctostaphylos uva-ursi.

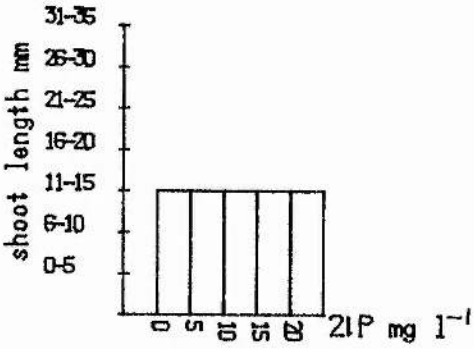


Fig 94 . Erica carnea.

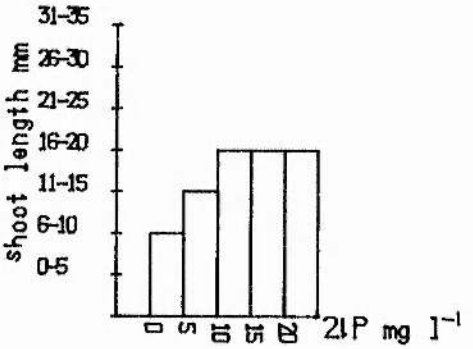


Fig 95 . Gaultheria hispidula.

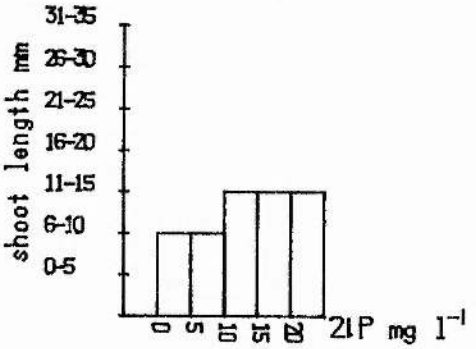


Fig 96 . Kalmia angustifolia.

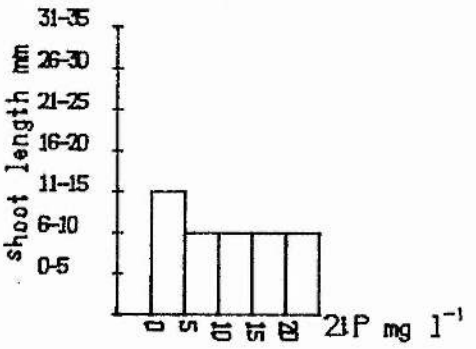


Fig 97 . Rhododendron arboreum.

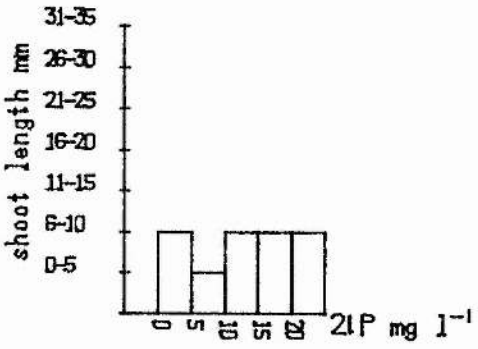


Fig 98 . Rhododendron chamaethomsonii.

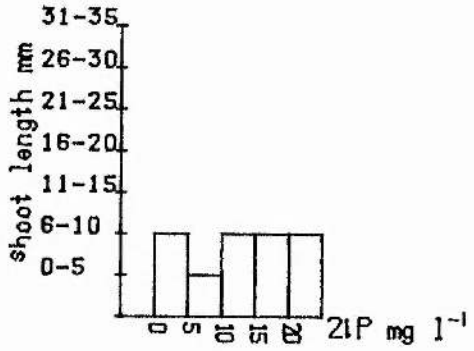


Fig 99 . Rhododendron chikor.

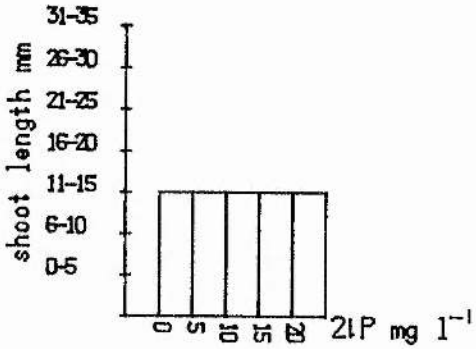


Fig 100. Rhododendron chinsayii.

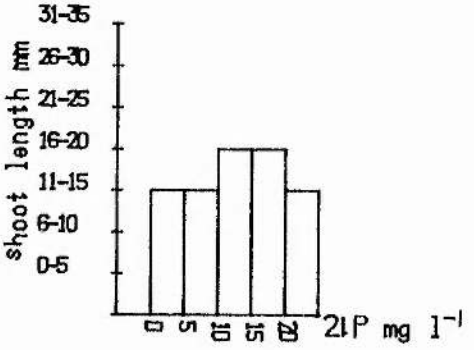


Fig 101 . Rhododendron dauricum .

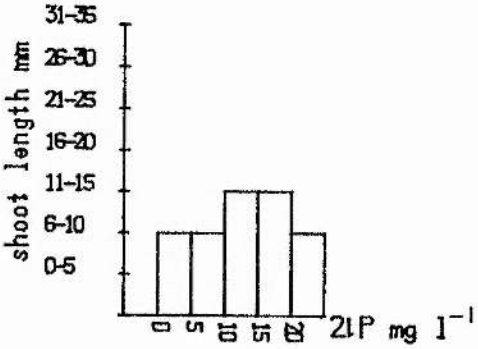


Fig 102. Rhododendron fastigiatum.

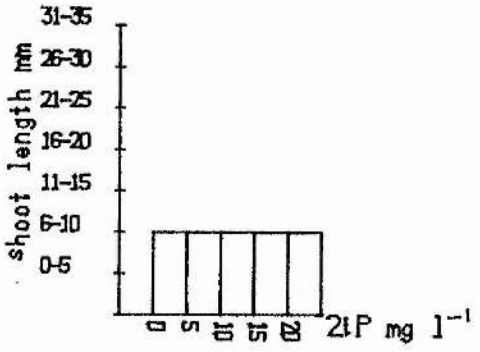


Fig 103. Rhododendron forrestii.

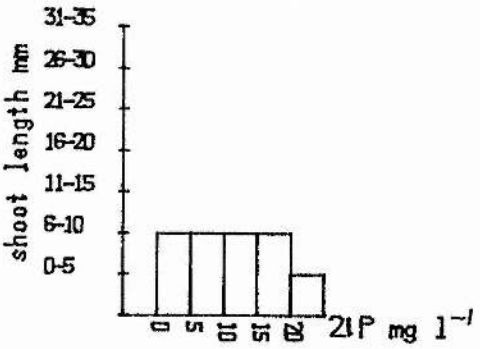


Fig 104. Rhododendron kelskei.

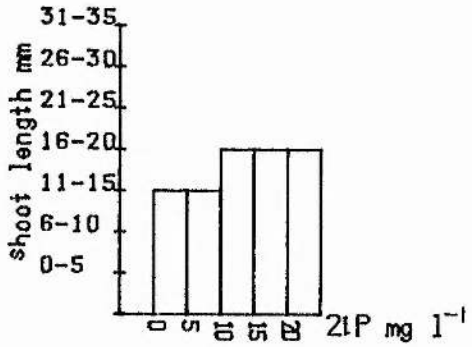


Fig 105. Rhododendron leucaspis.

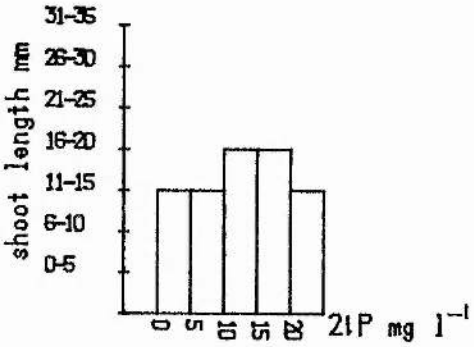


Fig 106. Rhododendron lutescens.

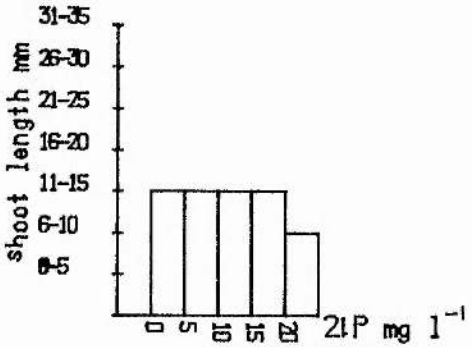


Fig 107. Rhododendron PJM'Victor.

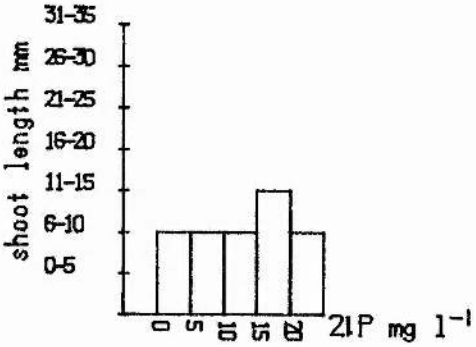


Fig 108. Rhododendron racemosum.

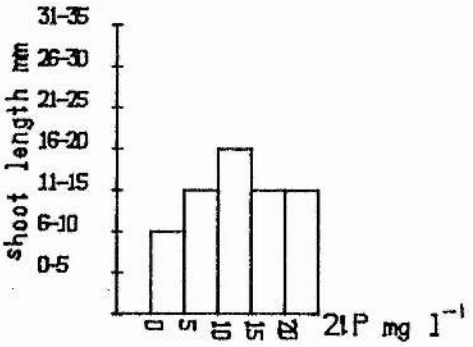


Fig 109. Rhododendron 'vuyks'.

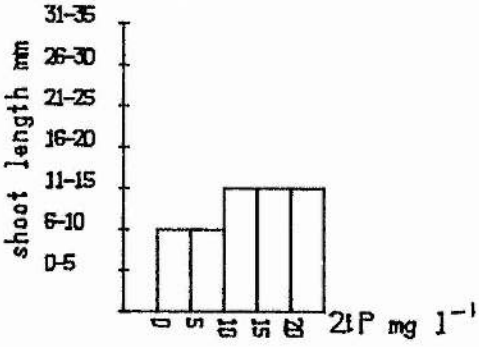


Fig 116. Rhododendron williamsianum.

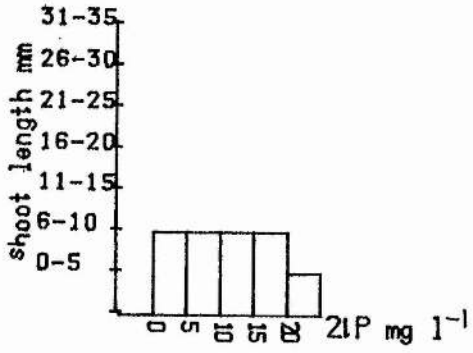


Fig 117. Vaccinium vitis-idaea.

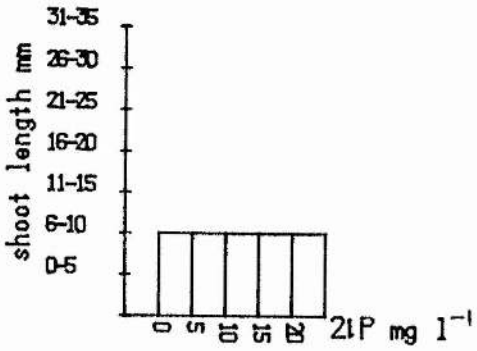


Table 12.

Comparison of shoot length in cultures producing maximal shoot number on BA medium with shoot length in cultures producing maximal shoot number on 2iP medium : Rosaceae.

Species	BA shoot length as% 2iP shoot length	p
Chaenomeles japonica	66.3	<.001
Cotoneaster dammeri	58.1	<.001
Crataegus brachyacantha	71.0	<.001
Crataegus 'Toba'	63.8	<.001
Potenti lla 'Coronation Triumph'	82.1	<.001
Potentilla 'Sutter's Gold'	90.5	<.001
Prunus cerasifera	66.0	<.001
Prunus tomentosa	44.4	<.001
Pyracantha coccinea	73.6	<.001
Spiraea 'Froebelii'	74.6	<.001

Significance was determined using a t test.

Table 13.

Comparison of shoot length in cultures producing maximal shoot number on BA medium with shoot length in cultures producing maximal shoot number on 2iP medium : Ericaceae.

Species	BA shoot length as% 2iP shoot length	p
Arctostaphylos media	44.4	<.001
Arctostaphylos uva-ursi	65.5	<.001
Erica carnea	16.7	<.001
Kalmia angustifolia	100.9	N.S.
Rhododendron arboreum	40.2	<.001
Rhododendron chamae-thomsonii	37.7	<.001
Rhododendron 'Chikor'	62.4	<.001
Rhododendron fastigiatum	37.5	<.001
Rhododendron forrestii	98.1	N.S.
Rhododendron keiskei	44.8	<.001
Rhododendron leucaspis	72.2	<.001
Rhododendron lutescens	97.7	N.S.
Rhododendron 'P.J.M.Victor'	61.5	<.001
Rhododendron racemosum	46.3	<.001
Rhododendron 'Vuyk's'	23.1	<.001
Rhododendron williamsianum	34.3	<.001
Vaccinium vitis-idaea	35.7	<.001

Significance was determined using a t test.

Table 14. Mean length increase (mm) of initial shoot explant in four weeks on BA medium: Rosaceae

Species	BA concentration (mg l^{-1})							Linear correlation (r) p
	0	0.1	0.5	1.0	2.5	5.0	10.0	
Chaenomeles japonica	13.0	11.0	7.8	6.0	4.8	2.8	1.3	-.7740 (p<.05)
Cotoneaster dammeri	27.5	24.3	19.5	17.5	15.3	6.5	3.8	-.9089 (p<.01)
Crataegus brachyacantha	17.3	14.5	13.8	9.8	5.3	2.8	2.5	-.8325 (p<.05)
Crataegus 'Toba'	11.0	8.0	5.8	4.5	3.5	1.5	1.3	-.7737 (p<.05)
Potentilla 'Coronation Triumph'	13.0	4.3	3.8	3.0	1.5	0.5	0.8	-.8506 (p<.02)
Potentilla 'Sutter's Gold'	9.55	6.8	5.8	4.0	3.0	1.3	1.0	-.7993 (p<.05)
Prunus cerasifera	26.0	21.0	20.3	11.0	4.5	3.5	3.3	-.7608 (p<.05)
Prunus tomentosa	17.8	13.0	12.3	12.5	9.3	4.0	4.0	-.8639 (p<.02)
Pyracantha coccinea	12.0	8.0	7.0	6.5	5.8	2.8	2.0	-.8322 (p<.05)
Spiraea 'Froebelii'	16.5	7.0	3.8	3.0	3.0	2.5	2.5	-.4804 (N.S.)

Table 15.Mean length increase (mm) of initial shoot explant in four weeks on 2iP medium:

Rosaceae

Species	2iP concentration (mg l^{-1})					Linear correlation (r)
	0	5.0	10.0	15.0	20.0	
Chaenomeles japonica	13.0	12.8	12.8	12.8	9.8	-.5436 (p<.02)
Cotoneaster dammeri	27.5	26.8	25.3	23.8	21.8	-.7985 (p<.001)
Crataegus brachyacantha	17.3	16.0	15.5	14.5	10.0	-.7090 (p<.001)
Crataegus 'Toba'	11.0	11.0	10.0	9.8	6.3	-.7383 (p<.001)
Potentilla 'Coronation Triumph'	13.0	13.0	14.5	11.3	6.8	-.6605 (p<.01)
Potentilla 'Sutter's Gold'	9.55	17.0	12.3	9.5	8.8	-.3939 (N.S.)
Prunus cerasifera	26.0	25.0	24.5	25.0	15.3	-.7064 (p<.001)
Prunus tomentosa	17.8	15.5	19.0	20.3	14.0	-.1438 (N.S.)
Pyracantha coccinea	12.0	11.5	8.3	6.3	5.3	-.1244 (NS.)
Spiraea 'Froebelii'	16.5	12.3	7.8	8.5	5.5	-.8861 (p<.001)

Table 16.Mean length increase (mm) of initial shoot explant in four weeks on BA medium:

Ericaceae

Species	BA concentration (mg l^{-1})							Linear correlation (r)
	0	0.1	0.5	1.0	2.5	5.0	10.0	
Arctostaphylos media	11.5	9.3	6.3	6.0	5.3	1.5	0.3	-.8488 (p<.001)
Arctostaphylos uva-ursi	11.8	11.3	9.8	9.0	6.8	3.5	1.0	-.9160 (p<.001)
Erica carnea *	9.5	3.3	-	-	-	-	-	-
Kalmia angustifolia	12.8	11.8	8.8	7.0	2.8	2.0	0.3	-.8136 (p<.001)
Rhododendron arboreum	8.3	7.8	6.0	3.3	2.8	0.5	0	-.8303 (p<.001)
Rhododendron chamae-thomsonii	7.0	6.5	6.5	5.8	3.5	0.3	0	-.9503 (p<.001)
Rhododendron 'Chikor'	10.0	8.8	2.8	1.5	1.5	1.0	0.8	-.5882 (p<.01)
Rhododendron fastigiatum *	8.8	3.0	-	-	-	-	-	-
Rhododendron forrestii	8.0	6.8	6.0	4.5	2.3	2.0	1.0	-.8162 (p<.001)

Table 16 continued

Rhododendron keiskei	14.3	13.3	10.8	9.8	7.8	4.8	1.3	-.9055 (p<.001)
Rhododendron leucaspis	13.3	11.0	8.0	4.0	3.3	2.8	3.0	-.6437 (p<.001)
Rhododendron lutescens	18.0	17.31	15.0	11.0	5.8	3.8	3.0	-.8294 (p<.001)
Rhododendron 'P.J.M.Victor' *	16.5	6.0	-	-	-	-	-	-
Rhododendron racemosum	15.0	14.0	11.8	10.5	3.5	2.5	2.8	-.7933 (p<.001)
Rhododendron 'Vuyk's'	14.8	13.3	9.5	3.8	3.3	3.5	2.0	-.6781 (p<.001)
Rhododendron williamsianum	7.8	7.5	3.3	1.8	2.5	1.8	0	-.7004 (p<.001)
Vaccinium vitis-idaea	7.0	7.5	3.0	2.8	3.5	1.8	0.5	-.7528 (p<.001)

* species in which BA treatments greater than 0.1 mg l^{-1} were toxic

Table 17. Mean length increase (mm) of initial shoot explant in four weeks on 2iP medium : Ericaceae

Species	2iP concentration (mg l^{-1})				
	0	5.0	10.0	15.0	20.0
Arctostaphylos media	11.5	11.5	13.3	8.8	6.0
Arctostaphylos uva-ursi	11.8	12.0	11.5	15.3	10.5
Erica carnea	9.5	9.8	10.5	11.8	7.8
Gaultheria hispidula	10.5	13.5	12.8	13.5	8.5
Kalmia angustifolia	12.8	8.5	8.3	9.0	5.8
Rhododendron arboreum	8.3	7.0	7.8	5.8	3.8
Rhododendron chamae-thomsonii	7.0	7.0	8.3	9.0	5.5
Rhododendron 'Chikor'	10.0	9.8	11.8	11.0	7.5
Rhododendron chinsayii	8.0	8.5	8.8	9.8	6.3

Table17 continued.

Rhododendron dauricum	15.0	15.3	12.8	18.0	7.5
Rhododendron fastigiatum	8.8	8.5	9.0	9.5	6.8
Rhododendron forrestii	8.0	8.5	9.3	7.0	3.8
Rhododendron keiskei	14.3	14.8	17.0	9.3	8.5
Rhododendron leucaspis	13.3	14.0	14.5	15.8	10.8
Rhododendron lutescens	18.0	16.8	17.3	18.5	9.5
Rhododendron 'P.J.M.Victor'	16.5	16.8	19.0	19.8	10.5
Rhododendron racemosum	15.0	15.0	15.5	11.3	6.3
Rhododendron 'Vuyk's'	14.8	15.0	11.3	17.0	7.0
Rhododendron williamsianum	7.8	7.3	6.8	5.8	4.0
Vaccinium vitis-idaea	7.0	7.3	9.8	11.3	4.5

A significant linear relationship was not demonstrated for this data set.

Table 18.

Comparison of shoot length increase (initial explant) on BA medium with increase on 2 iP medium : Rosaceae

Species	BA shoot length as% 2iP shoot length	p
Chaenomeles japonica	37.3	<.001
Cotoneaster dammeri	69.3	<.001
Crataegus brachyacantha	62.9	<.001
Crataegus 'Toba'	46.2	<.001
Potentilla 'Coronation Triumph'	29.3	<.001
Potentilla 'Sutter's Gold'	33.8	<.001
Prunus cerasifera	81.0	<.001
Prunus tomentosa	45.7	<.001
Pyracantha coccinea	69.7	<.001
Spiraea 'Froebelii'	48.4	<.001

Significance was determined using a t test.

Table 19.

Comparison of mean shoot length increase (initial explant) on BA medium with increase on 2iP medium in maximal shoot number cultures : Ericaceae

Species	BA shoot length as% 2iP shoot length	p
Arctostaphylos media	45.3	<.001
Arctostaphylos uva-ursi	23.0	<.001
Erica carnea	27.7	<.001
Kalmia angustifolia	30.6	<.001
Rhododendron arboreum	52.2	<.001
Rhododendron chamae-thomsonii	2.8	<.001
Rhododendron 'Chikor'	19.3	<.001
Rhododendron fastigiatum	31.6	<.001
Rhododendron forrestii	24.3	<.001
Rhododendron keiskei	27.9	<.001
Rhododendron leucaspis	25.4	<.001
Rhododendron lutescens	31.1	<.001
Rhododnedron 'P.J.M.Victor'	30.4	<.001
Rhododendron racemosum	22.6	<.001
Rhododendron 'Vuyk's'	22.1	<.001
Rhododendron williamsianum	48.1	<.001
Vaccinium vitis-idaea	26.7	<.001

Significance was determined using a t test.

Table 20.

Origin of shoots produced on BA and 2iP medium : Rosaceae

Species	BA		2iP	
	Ax	Ad	Ax	Ad
Chaenomeles japonica	*	-	*	-
Cotoneaster dammeri	*	*	*	-
Crataegus brachyacantha	*	*	*	-
Crataegus 'Toba'	*	*	*	-
Potentilla 'Coronation Triumph'	*	*	*	*
Potentilla 'Sutter's Gold'	*	*	*	*
Prunus cerasifera	*	*	*	-
Prunus tomentosa	*	*	*	-
Pyracantha coccinea	*	-	*	*
Spiraea 'Froebelii'	*	*	*	*

Ax = axillary: Ad = adventitious

Table 21.

Origin of shoots produced on BA and 2iP medium :

Ericaceae

Species	BA		2iP	
	Ax	Ad	Ax	Ad
Arctostaphylos media	*	*	*	*
A. uva-ursi	*	*	*	*
Erica carnea	*	-	*	*
Gaultheria hispidula	-	-	*	-
Kalmia angustifolia	*	-	*	-
Rhododendron arboreum	*	-	*	-
R. Chamae-thomsonii	*	-	*	-
R. 'chikor'	*	-	*	*
R. chinsayii	-	-	*	-
R. dauricum	-	-	*	-
R. fastigiatum	*	-	*	-
R. forrestii	*	-	*	-
R. keikei	*	-	*	-
R. leucaspis	*	-	*	-
R. lutescens	*	-	*	-

R. P.J.M. Victor	*	-	*	-
R. racemosum	*	-	*	:
R. 'Vuyk's'	*	-	*	*
R. williamsianum	*	-	*	-
Vaccinium vitis-idaea	*	*	*	*

Ax = axillary: Ad = adventitious

* = source of origin

3.12 DYNAMICS OF IN VITRO SHOOT FORMATION

The previous section demonstrated a difference in shoot length bewteen BA and 2iP treated explants. It was hypothesised that this difference was due to the rate of formation of the shoots. This section tests this hypothesis.

Method

Experiment 1.

The following species were used in the experiment :- Rosaceae : Crataegus brachyacantha, Prunus cerasifera, Spiraea 'Froebelii'; Ericaceae : Arctostaphylos uva-ursi, Rhododendron 'chikor', Rhododendron 'PJM Victor'. BA or 2iP were incorporated in the nutrient medium at the concentrations which induced maximal shoot proliferation in Section 3.11.

Shoot number was recorded at the end of 1, 2, 3, 4, 5, 6, 7 and 8 weeks incubation in light (16 hour photoperiod). Number of primary and secondary shoots was noted after 8 weeks incubation.

Experiment 2.

Species used for this experiment were Spiraea 'Froebelii' (Rosaceae) and Arctostaphylos uva-ursi (Ericaceae). BA or 2iP was incorporated in the nutrient medium at the following concentrations :- BA : 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0; 2iP : 0, 5.0, 10.0, 15.0, 20.0 mg 1-1.

Shoot number was recorded at the end of 1, 2, 3 and 4 weeks of incubation.

Experiment 3.

Species tested in this experiment were Spiraea 'Froebelii', Crataegus brachyacantha (Rosaceae), Arctostaphylos uva-ursi, and Rhododendron 'chikor' (Ericaceae). BA and 2iP were supplied in the nutrient medium at the concentrations which induced maximal shoot proliferation (4.1) for 1, 3, 5, 7, 14, 21 or 28 days. Explants were then placed on nutrient medium without growth regulators.

Shoot number and length were recorded at the end of a four-week total incubation period.

Results

Experiment 1.

Mean shoot number formed per week is given in Tables 22 and 23. On BA medium, most shoots formed in the second week of culture while on medium containing 2iP, most shoots formed in the third week. On BA medium,, a second flush of shoots was formed in the fifth and sixth weeks. Table 25 shows these effects calculated over all of the species tested. An analysis of variance (Table 24) showed a significant effect on shoot formation due to period of culture ($p<.001$), a significant difference bewteen BA and 2iP ($p<.001$) and a significant interaction between time of culture and growtjh regulator ($p<.001$).

Number of shoots with secondary branches is given in Tables 26 and 27. Secondary branches were formed between the fifth and eighth week in culture (Table 28). Secondary branching occurred to a greater extent in BA treatments than in 2iP treatments.

Experiment 2.

Percentage of the total shoot number after four weeks is given for each week in Tables 29 to 32. An analysis of variance showed a significant interaction between time for shoots to form and concentration ($p<.001$) (Table 33). When BA or 2iP concentration was low, shoots took longer to form. This was true for both BA and 2iP and for both species tested.

Experiment 3.

Figures 118-125 give mean shoot number after different exposure periods to BA and 2iP. A significant effect ($p<.001$) was shown due to exposure period and species (Tables 34 and35). Shoot number increased with duration of exposure period to both BA and 2iP in all species. Only a very few shoots were formed when the exposure period was 3 days or less. In all species, a longer exposure period to 2iP than to BA was necessary to produce the same number of shoots. This is summarized in Table 36.

Modal shoot length was plotted against duration of exposure to BA (Figures 126 to 129) and 2iP (Figures 130 to 133). Exposure period did not affect shoot length.

Table 22. Mean shoot number formed per week on medium containing BA

Species	Week Number									
	0	1	2	3	4	5	6	7	8	Mean
Crataegus brachyacantha	0	3.25	3.00	2.75	1.00	4.75	3.75	2.50	.25	2.36
Prunus cerasifera	0	1.50	2.75	1.25	.50	4.25	3.5	.75	.75	1.69
Spiraea 'Froebelii'	0	7.25	15.0	7.75	7.75	4.00	2.75	.75	.75	5.11
Arctostaphylos uva-ursi	0	1.25	9.25	3.75	1.25	4.25	4.50	2.00	.75	3.00
Rhododendron 'Chikor'	0	.25	.25	.75	.25	.25	1.00	0	0	.306
Rhododendron P.J.M. Victor	0	0	.25	.50	0	0	0	0	0	.0803
Mean Week	0	2.25	5.08	2.79	1.79	2.92	2.58	1.00	.417	

L.S.D. Week Means = 0.533

L.S.D. Species Means = 0.435

L.S.D. Means in body of table = 1.31

Table 23 Mean shoot number formed per week on medium containing 2iP

Species	Week Number								
	0	1	2	3	4	5	6	7	8	Mean
Crataegus brachyacantha	0	2.25	.50	1.50	.75	.50	0	0	.50	.667
Prunus cerasifera	0	1.50	1.00	1.25	.25	.50	.75	1.00	.50	0.75
Spiraea 'Froebelii'	0	3.50	7.50	14.50	5.00	.75	1.00	.75	.75	3.75
Arctostaphylos uva-ursi	0	.50	1.75	2.00	3.25	.25	0	0	0	.861
Rhododendron 'Chikor'	0	.50	1.75	7.75	.25	.25	0	0	0	1.17
Rhododendron P.J.M. Victor	0	.25	1.50	4.50	0	0	0	0	0	.694
Mean Week	0	1.42	2.33	5.25	1.58	.375	.292	.292	.292	

L.S.D. Week Means = 0.533

L.S.D. Species Means = 0.435

L.S.D. Means in body of table = 1.31

Table 24.

Analysis of variance for data presented in Tables 22 and 23.

SOURCE	S.S.	D.F.	M.S.	F	p
Weeks	735.459	8	91.931	112.399	<.001
Species	750.241	5	150.048	183.455	<.001
Growth Regulator	65.333	1	65.333	79.879	<.001
Weeks * Variety	933.634	40	23.341	28.538	<.001
Weeks * Growth Regulator	253.542	8	31.693	38.749	<.001
Variety * Growth Regulator	138.167	5	27.633	33.786	<.001
Weeks * Variety * Growth Regulator	310.708	40	7.768	7.750	<.001
Error	265.000	324	0.8179		
Total	3452.074	431			

Table 25.

Mean shoot number formed per week on medium containing BA or 2iP calculated over all species tested.

WEEK	BA SHOOT NUMBER	2iP SHOOT NUMBER
0	0e	0d
1	2.25c	1.42c
2	5.08a	2.33b
3	2.79b	5.25a
4	1.79cd	1.58c
5	2.92b	.375d
6	2.58bc	.292d
7	1d	.292d
8	.417e	.292d

Data followed by different letters within a column at significantly different ($p<.05$).

Table 26.

Number of primary and secondary shoots formed in four and eight weeks on BA medium.

Species	Shoots:-	Primary		Secondary	
Weeks		4	8	4	8
Crataegus brachyacantha		10.0	14.5	0	6.75
Prunus cerasifera		6.0	9.25	0	6.0
Spiraea 'Froebelii'		37.75	38.75	0	7.25
Arctostaphylos uva-ursi		15.5	18.75	0	8.25
Rhododendron 'chikor'		1.5	1.75	0	1.0
Rhododendron 'P.J.M.Victor'		0.75	0.75	0	0

Table 27.

Number of primary and secondary shoots formed in four and eight weeks on 2iP medium.

Species	Shoots:-	Primary		Secondary	
		4 weeks	8 weeks	4 weeks	8 weeks
Crataegus brachyacantha		5.0	6.0	0	0
Prunus cerasifera		4.0	6.25	0	0.5
Spiraea 'Froebelii'		29.25	32.50	1.25	1.25
Arctostaphylos uva-ursi		7.5	7.75	0	0
Rhododendron 'chikor'		10.25	10.50	0	0
Rhododendron 'P.J.M.Victor'		6.25	6.25	0	0

Table 28.

Mean number of primary and secondary shoots calculated over all species tested.

Shoot type and week number	BA Shoot number	2iP Shoot number
4 weeks primary	2.02b	2.21a
8 weeks primary	2.20a	2.32a
4 weeks secondary	0d	.132b
8 weeks secondary	1.49c	.190b

Data followed by different letters within a column at significantly different ($p<.05$).

Table 29.

Total number of shoots formed (recorded once per week) in Spiraea 'Froebelii' on BA medium.

Week	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
week 1	0	2.04	19.21	24.81	27.21	25.45	33.33
week 2	0	15.31	39.74	49.61	41.18	43.36	44.00
week 3	0	65.31	20.53	22.48	19.12	16.36	14.67
week 4	100.0	17.35	20.53	15.50	12.50	11.82	8.00

Table 30.

Total number of shoots formed (recorded once per week) in Spiraea 'Froebelii' on 2iP medium.

Week	2iP concentration (mg l^{-1})				
	0	5.0	10.0	15.0	20.0
week 1	0	0	11.48	17.89	41.86
week 2	0	0	24.59	23.16	34.88
week 3	0	34.00	47.54	35.79	16.28
week 4	100.0	66.00	16.39	23.16	6.98

Table 31.

Total number of shoots formed (recorded once per week) in Arctostaphylos uva-ursi on BA medium.

Week	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
week 1	0	0	0	16.67	15.38	8.06	60.00
week 2	0	0	5.89	22.22	15.38	59.68	15.00
week 3	60.00	33.33	70.59	44.44	53.85	24.19	15.00
week 4	40.00	66.67	23.53	16.67	15.38	8.06	10.00

Table 32.

Total number of shoots formed (recorded once per week) in Arctostaphylos uva-ursi on 2iP medium.

Week	2iP concentration (mg l^{-1})				
	0	5.0	10.0	15.0	20.0
week 1	0	0	0	6.67	20.00
week 2	0	0	9.09	23.33	60.00
week 3	60.00	42.86	45.45	26.67	13.33
week 4	40.00	57.14	45.45	43.33	6.67

Table 33.

Analysis of variance for data presented in Tables 29 to 32.

SOURCE	S.S.	D.F.	M.S.	F	P
BA	5034.804	6	839.134	200.177	<.001
Weeks	4726.621	3	1575.540	375.845	<.001
Species	9712.611	1	9712.611	2316.956	<.001
BA * week	1108.911	18	61.606	14.696	<.001
BA * species	2887.732	6	481.289	114.812	<.001
Week * species	1710.906	3	570.302	136.047	<.001
BA * week * species	685.125	18	38.063	38.271	<.001
Error	704.250	168	4.192		
Total	26570.960	223			

Table 33 continued.

Analysis of variance for data presented in Tables 29 to 32.

SOURCE	S.S.	D.F.	M.S.	F	P
2iP	2050.288	4	512.572	307.928	<.001
Weeks	1765.269	3	588.423	353.496	<.001
Species	1870.056	1	1870.056	1123.438	<.001
2iP * week	830.7321	12	69.230	41.590	<.001
2iP * species	1189.038	4	297.259	170.579	<.001
Week * species	527.0696	3	175.690	105.546	<.001
2iP * week * species	401.713	12	33.476	59.526	<.001
Error	199.7500	120	1.665		
Total	8833.944	159			

Figures 118 to 125.

Mean shoot number after varying exposure periods to BA or 2iP.

Fig118. Crataegus brachyacantha BA

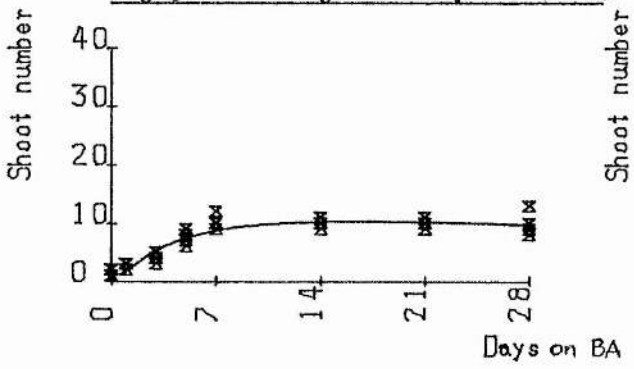


Fig119. Spiraea 'Froebelii' BA.

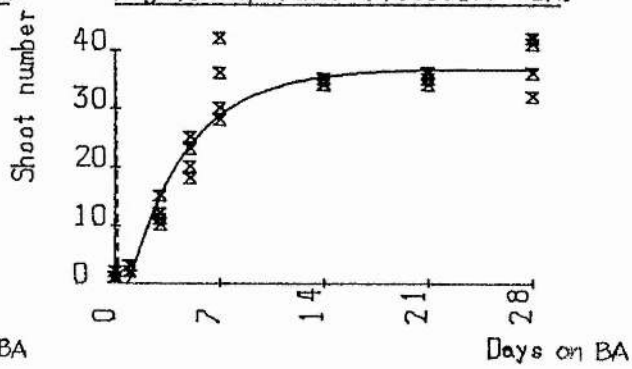


Fig120. Crataegus brachyacantha 2iP.

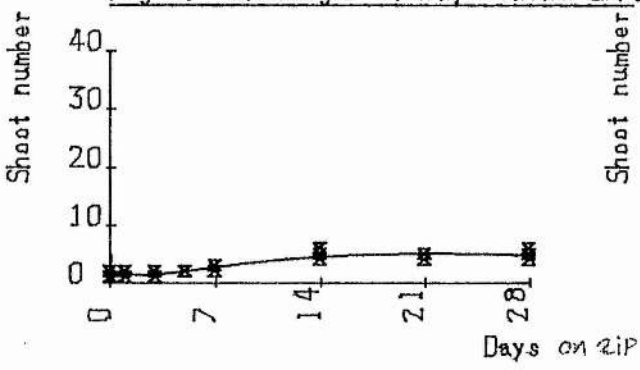


Fig121. Spiraea 'Froebelii' 2iP.

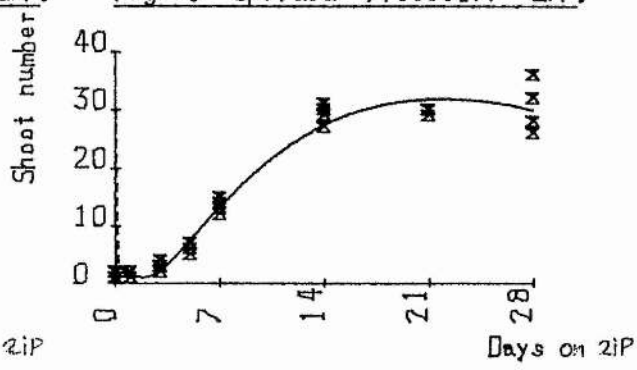


Fig 122. Arctostaphylos uva-ursi BA

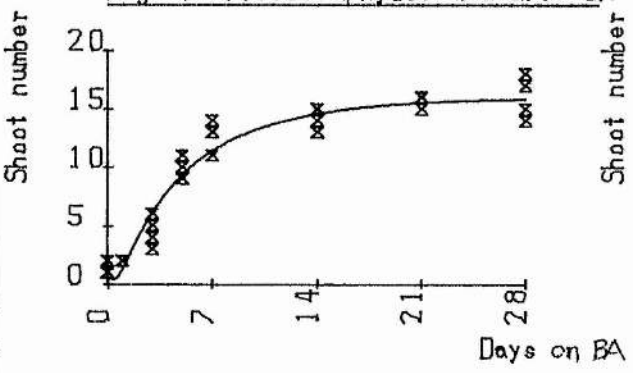


Fig 123 Rhododendron chikor BA.

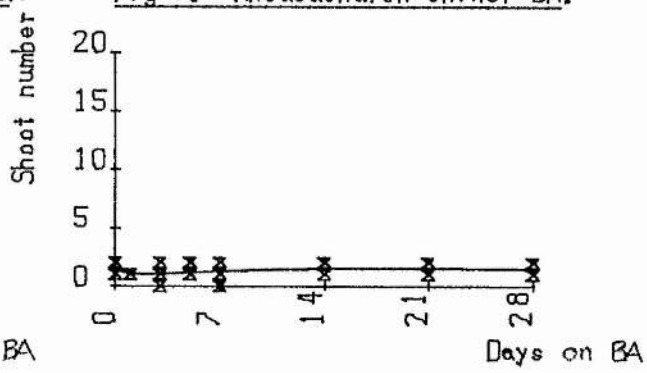


Fig 124 Arctostaphylos uva-ursi 2iP.

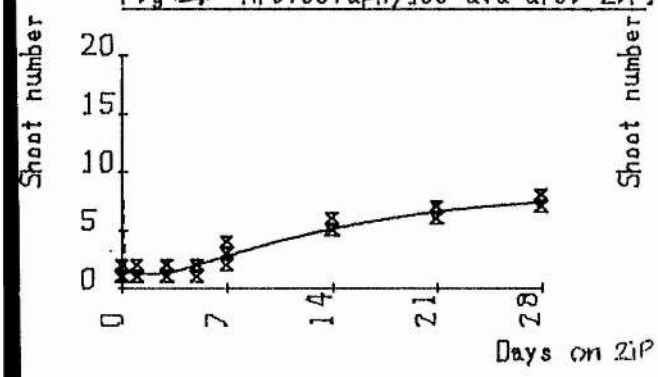


Fig 125 Rhododendron chikor 2iP.

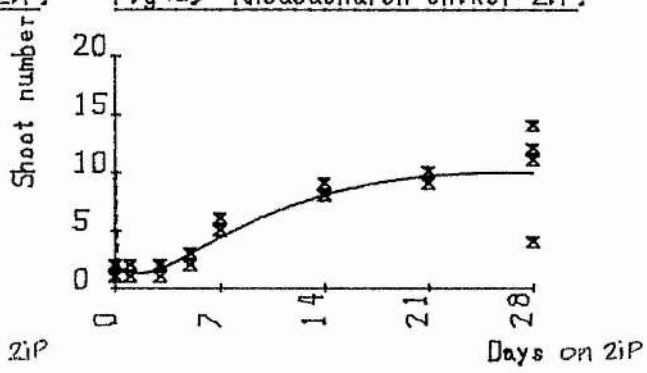


Table 34.

Analysis of variance for data in Figures 118-125 BA.

SOURCE	S.S.	D.F.	M.S.	F	p
Days	4396.250	7	628.036	199.972	<.001
Species	7565.313	3	2521.771	802.952	<.001
Interaction	3498.813	21	166.610	53.050	<.001
Error	301.500	96	3.141		
Total	15761.875	127			

Table 35.

Analysis of variance for data in Figures 118-125 2iP.

SOURCE	S.S.	D.F.	M.S.	F	p
Days	3167.969	7	452.567	271.540	<.001
Species	2727.531	3	909.177	545.506	<.001
Interaction	2520.219	21	120.010	72.006	<.001
Error	160.000	96	1.667		
Total	8575.719	127			

Table 36.

Mean shoot number calculated over all species tested after varying incubation periods on medium containing BA or 2iP.

days	shoot no.	
	2iP	BA
0	1.38e	1.38e
1	1.38e	2.06e
3	1.94e	5.38d
5	3.06d	10.06c
7	6.06c	14.56b
14	12b	14.94b
21	12b	15.56ab
28	13.31a	16.31a

Data followed by different letters within columns are significantly different from each other ($p<.05$).

Figures 126 to 133.

Modal shoot length after varying exposure periods to BA or 2iP.

Fig 126. Crataegus brachyacantha.

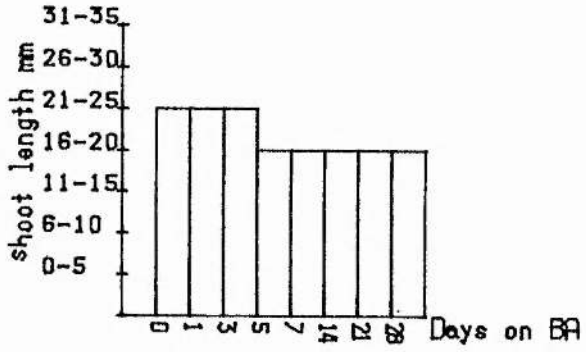


Fig 127. Spiraea 'Froebellii'.

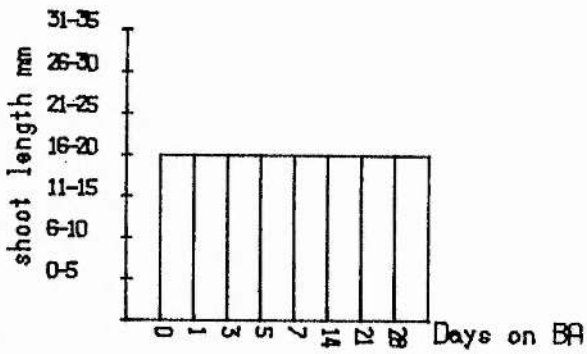


Fig 128. Arctostaphylos uva-ursi.

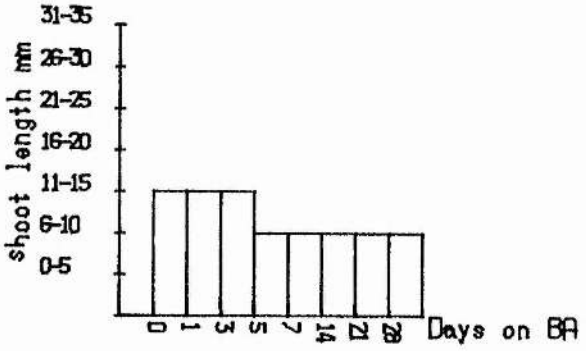


Fig 129. Rhododendron chikor.

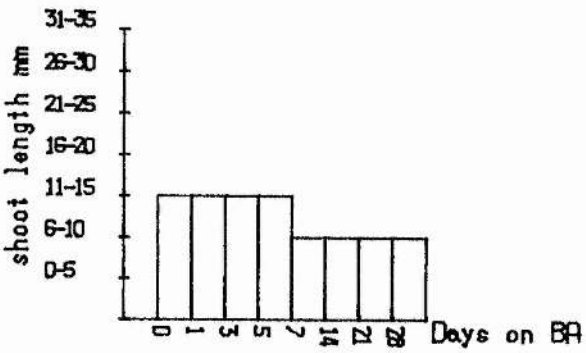


Fig 130. Crataegus brachyacantha.

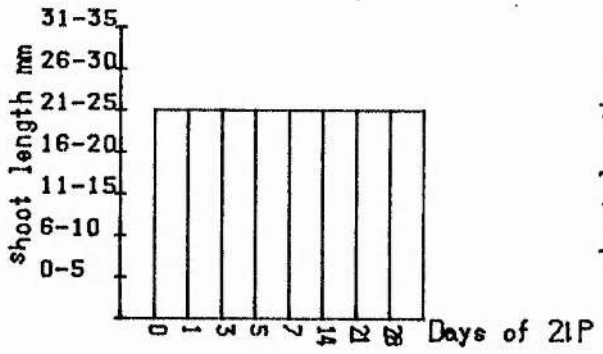


Fig 131. Spiraea 'Froebellii'.

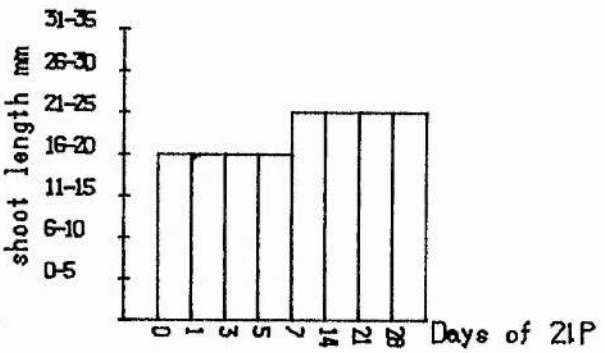


Fig 132. Arctostaphylos uva-ursi.

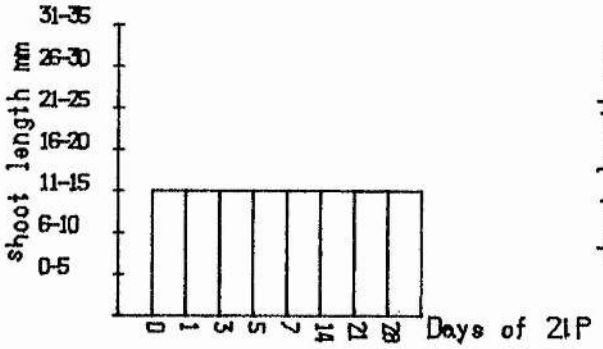
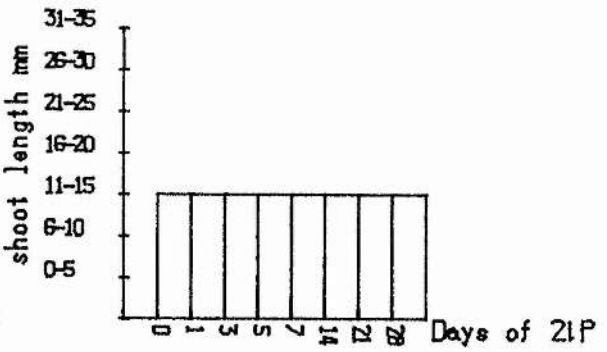


Fig 133. Rhododendron chlkor.



3.13 SHOOT FORMATION IN INTACT PLANTS

Method

Experiment 1.

Intact one year old plants of the following species were used in the experiment :- Potentilla 'Coronation Triumph', Spiraea 'Froebelii', Arctostaphylos uva-ursi, Rhododendron 'PJM Victor'. The following treatments were given :-

1. 200 ppm BA
2. 200 ppm BA + 1% DMSO
3. H_2O
4. H_2O + 1% DMSO

A few drops of detergent as a wetting agent were added to each solution. Plants (4 replicates per treatment) were sprayed until run-off twice per week.

Plants were incubated at a temperature of 25°C and were illuminated for 16 hours per day. Number of new shoots formed was recorded after 7, 14, 21 and 28 days.

Experiment 2.

Species used were Spiraea 'Froebelii' (Rosaceae) and Arctostaphylos uva-ursi. One spray only of

cytokinin + DMSO was given. BA and 2iP concentrations used were 0, 100, 200, 300, 400, 500, 1000, 1500 and 2000 ppm. Shoot number was recorded after 1, 2, 3, 4, 5, 6, 7 and 8 weeks incubation as described in Experiment 1. above. Shoot length was recorded after 8 weeks incubation.

Results

Experiment 1.

Mean number of shoots formed is given in Table 37. A significantly greater number of shoots was formed on BA treated plants than on control plants in Spiraea (Plate 5) and Arctostaphylos (Table 37) but there was no significant increase in shoot number in Rhododendron and Potentilla but when results for the different species were combined, a significant difference was demonstrated between control and BA treatments ($p<.001$) (Table 37). There was also a significant difference between BA and BA + DMSO treatments ($p<.001$) - BA + DMSO gave more shoots. An analysis of variance (Table 39) showed a significant difference in shoot number due to treatment ($p<.001$)

and weeks of treatment ($p<.001$) with weeks of culture ($p<.001$) (most shoots formed in weeks one and two), a significant difference due to species ($p<.001$) and a significant interaction.

Experiment 2.

Mean new shoot number 8 weeks after treatment with BA or 2iP is given in Figures 134 tp 137. An analysis of variance (Table 40)showed that there was a significant effect due to concentration ($p<.001$) in both BA and 2iP treatments, a significant difference in shoot number between BA and 2iP treatments ($p<.001$), and a significant difference in shoot number between species ($p<.001$). Higher concentrations of 2iP (1500 ppm) than of BA (500 ppm) were necessary to promote maximal shoot formation (Table 41).

Modal shoot length 8 weeks after treatment was plotted against cytokinin concentration (Figures 138 to 141). Shoot length decreased with increasing BA concentration in both species, but 2iP concentration had no effect on shoot length.

Mean number of shoots formed per week is given in Figures 142 to 145 and Tables 42 to 45. Most shoots were formed between weeks 2 and 4 (Table 46). An analysis of variance showed a significant effect due to

concentration ($p<.001$) and a significant effect of species ($p<.001$) on shoot number (Table 47). A second flush of shoots (new shoots not secondary branches) was formed in week 7 in BA treatments.

Origin of Shoots

All shoots were axillary in origin (Plate 6).

Table 37.

Mean shoot number increase per week after repeated spray treatment : Potentilla 'Coronation Triumph'.

WEEK	H_2O	H_2O+DMSO	BA	BA+DMSO
week 1	3.5	3.5	3.5	6.75
week 2	1.0	1.0	4.0	1.0
week 3	0	0	0	0
week4	0	0	0	0

L.S.D. = 1.74

Mean shoot number increase per week after repeated spray treatment : Spiraea 'Froebelii'.

WEEK	H_2O	H_2O+DMSO	BA	BA+DMSO
week 1	5.0	5.25	7.25	15.5
week 2	3.75	2.25	15.5	8.75
week 3	2.0	2.75	1.25	6.0
week4	1.0	0	3.0	8.0

L.S.D. = 1.74 (for comparing means within and between Tables ($p<.05$).

Table 37 continued.

Mean shoot number increase per week after repeated spray treatment : Arctostaphylos uva-ursi.

WEEK	H_2O	H_2O+DMSO	BA	BA+DMSO
week 1	0	0	0	2.0
week 2	0	0	0	3.75
week 3	0	0	6.0	15.0
week4	0	0	6.25	6.0

L.S.D. = 1.74

Mean shoot number increase per week after repeated spray treatment : Rhododendron 'P.J.M. Victor'.

WEEK	H_2O	H_2O+DMSO	BA	BA+DMSO
week 1	0	0	0	0
week 2	0	0	0	1.25
week 3	0	0	1.0	0
week4	0	0	0	0

L.S.D. = 1.74 (for comparing means within Table 37 ($p<.05$).

Table 38.

Mean shoot number 4 weeks after repeated spray treatment (calculated over all species).

Treatment	MEAN
1. H_2O	1.02
2. H_2O + DMSO	0.922
3. BA	2.98
4. BA + DMSO	4.62

L.S.D. = .43 ($p<.05$)

Table 39.

Analysis of variance for data presented in Table 37.

SOURCE	S.S.	D.F.	M.S.	F	p
Treatment	601.137	3	200.379	137.771	<.001
Week	106.512	3	35.504	24.411	<.001
Species	973.387	3	324.462	223.086	<.001
Treatment * weeks	120.629	9	13.403	9.215	<.001
Treatment * species	427.379	9	47.487	32.650	<.001
Week * species	712.129	9	79.125	54.403	<.001
Treatment * week * species	650.293	27	24.085	10.883	<.001
Error	279.250	192	1.454		
Total	3870.715	255			

Figures 134 to 137.
Mean shoot number on intact plants eight weeks after BA or 2iP treatment.

Figures 138 to 141.
Modal shoot length eight weeks after BA or 2iP treatment.

Fig 134. S. 'Froebelii BA.

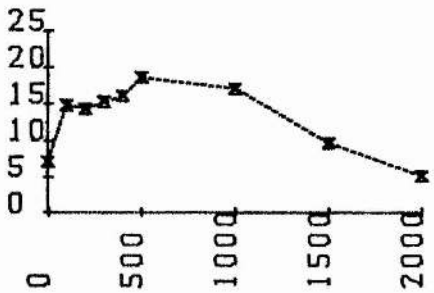


Fig 135. S. 'Froebelii' BA.

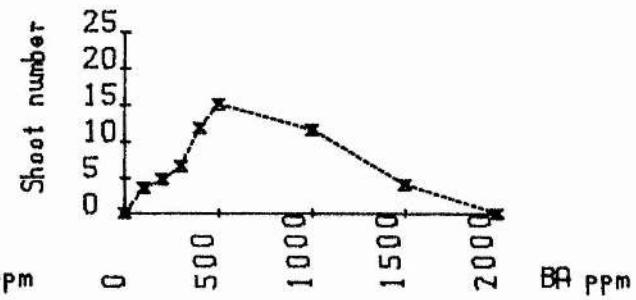


Fig 136. S. 'Froebelii 2iP.

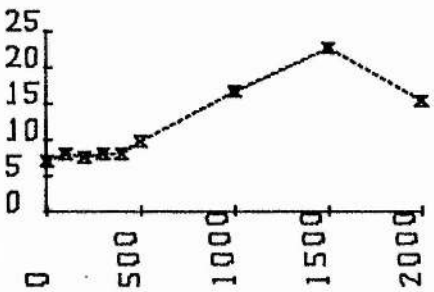


Fig 137. S. 'Froebelii' 2iP.

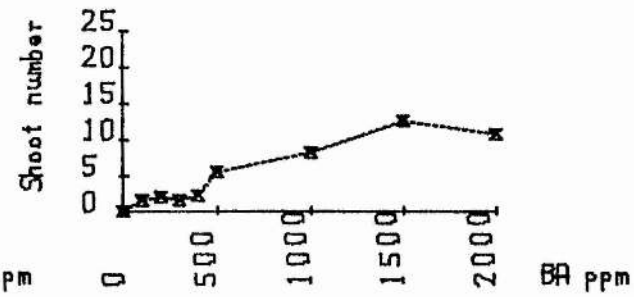


Fig138. Spiraea 'Froebellii'

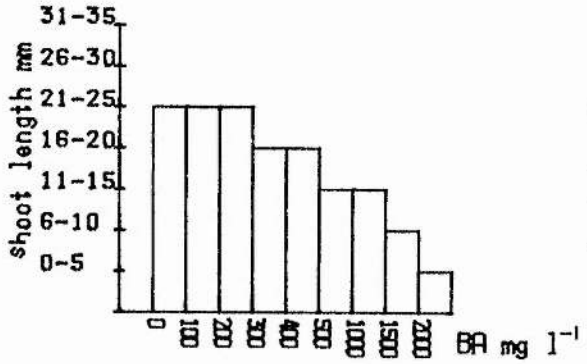


Fig139. Arctostaphylos uva-ursi.

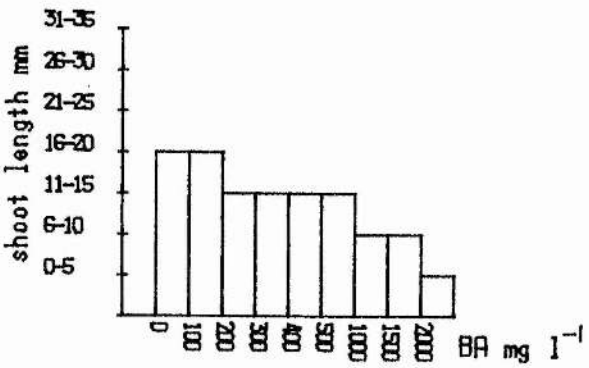


Fig140. Spiraea 'Froebellii'

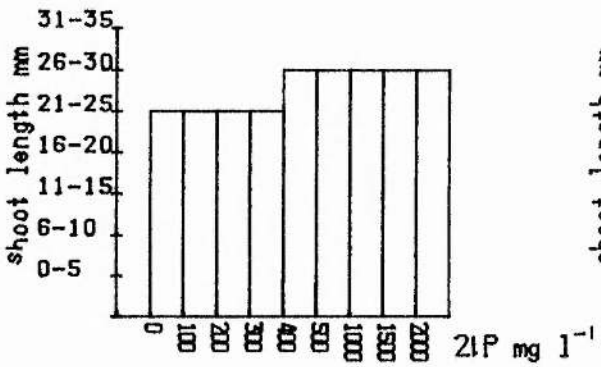


Fig141. Arctostaphylos uva-ursi.

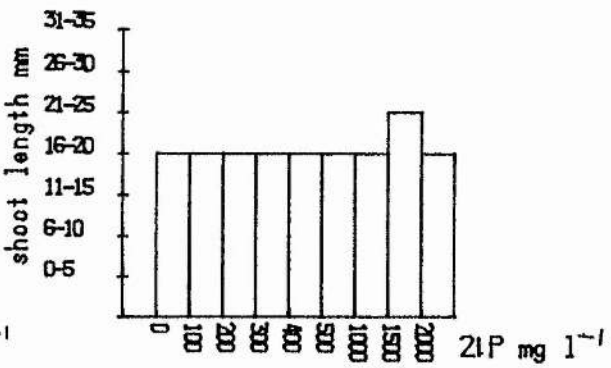


Table 40.

Analysis of variance for data presented in Figures 134 to 137.

SOURCE	S.S.	D.F.	M.S.	F	P
Concentration	1275.250	8	159.406	28.717	<.001
Species	1560.250	1	1560.250	281.079	<.001
BA / 2iP	84.028	1	84.028	15.138	<.001
Concentration * species	88.000	8	11.000	1.982	<.05
Concentarion * BA/2iP	1789.472	8	223.684	40.297	<.001
Species * BA/2iP	0.444	1	0.444	0.0801	N.S.
Concentration * species * BA/2iP	74.056	8	9.257	40.297	<.001
Error	599.500	108	5.551		
Total	5471.000	143			

Table 41.

Mean shoot number 8 weeks after BA or 2iP treatment (species combined).

CONCENTRATION ppm	BA	2iP
0	3.50	3.50
100	9.13	4.75
200	9.50	4.75
300	10.88	4.75
400	13.88	5.13
500	16.75	7.63
1000	14.25	12.38
1500	6.75	17.50
2000	2.50	13.00

L.S.D. = 2.40

Table 42.Mean shoot number formed per week after one BA spray treatment : Arctostaphylos uva-ursi.

BA concentration	WEEK NUMBER							
ppm	1	2	3	4	5	6	7	8
0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0.25	0
200	0	0	4.75	0	0	0.5	0	0
300	0	1.5	6.5	0	0	1.0	1.0	0
400	0.5	3.25	5.0	2.25	0	2.5	0	0
500	1.5	9.75	9.25	3.5	2.0	6.5	0	0
1000	1.0	11.25	6.25	1.5	0	7.75	0	0
1500	0.25	0	0	0	0	0	0	0
2000	0	0	0	0	0	0	0	0

Table 43.Mean shoot number formed per week after one 2iP spray treatment : Arctostaphylos uva-ursi.

2iP concentration	WEEK NUMBER							
ppm	1	2	3	4	5	6	7	8
0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0
200	0	0	0.25	0	0	0	0	0
300	0	0	0.5	0	0	0	0	0
400	0.75	0.5	2.0	1.0	0	0	0	0
500	0.5	0.5	1.0	3.0	0	0	0	0
1000	1.0	2.0	4.5	3.5	2.0	0	0	0
1500	1.0	4.75	4.25	3.0	0.5	0	0	0
2000	0	3.25	0	0	0	0	0	0

L.S.D. = 1.23

Table 44.Mean shoot number formed per week after one BA spray treatment : Spiraea 'Froebelii'.

BA concentration ppm	WEEK NUMBER 1	2	3	4	5	6	7	8
0	0	0	5.5	0	0	1.5	0	0
100	0	1.5	12.0	0	0	0.75	0	0.25
200	0	1.0	10.5	0	2.0	0.5	0.25	0
300	0	1.5	13.0	0	0.25	0	0.5	0
400	1.0	6.0	8.0	0	0	1.0	0	0
500	2.0	7.0	9.25	0	0	0.25	0	0
1000	0	2.0	14.25	0.25	0.25	0.25	0	0
1500	0	3.5	1.0	0	0	0	0	0
2000	0	0	0	0	0	0	0	0

Table 45.Mean shoot number formed per week after one 2iP spray treatment : Spiraea 'Froebelii'.

2iP concentration	WEEK NUMBER							
ppm	1	2	3	4	5	6	7	8
0	0	0	5.5	0	0	1.5	0	0
100	0	0	5.5	0	0	0	0	0
200	0	0	6.5	0.25	0	0	0	0
300	0	0	6.0	0	1.5	0	0	0
400	0	1.25	6.0	2.25	0	0	0	0
500	0	3.5	7.75	3.0	2.75	0	0	0
1000	0	4.5	12.5	4.5	0	0	0	0
1500	2.0	6.5	18.5	5.0	0.75	0	0	0
2000	0	4.0	11.25	3.5	1.0	0.25	0	0

L.S.D. = 1.23 (for comparing values within table or between Tables 42,43,44 and 45).

Table 46.Mean number of shoots formed per week (treatments combined).

Species	WEEK NUMBER							
	1	2	3	4	5	6	7	8
Arctostaphylos uva-ursi BA	0.361e	2.811b	3.417a	0.806d	0.222e	2.028c	0.139e	0e
Arctostaphylos uva-ursi 2iP	0.361b	1.222a	1.167a	1.167a	0.278b	0b	0b	0c
Spiraea 'Froebelii' BA	0.333cd	2.500b	8.167a	0.028d	0.278cd	0.472c	0.083d	0.28d
Spiraea 'Froebelii' 2iP	0.222cd	2.194b	8.833a	2.056b	0.667c	0.194d	0d	0d

Means within a row followed by a different letter are significantly different at the p.05 level.

Table 47.

Analysis of variance for data presented in Tables 42 to 45.

SOURCE	S.S.	D.F.	M.S.	F	p
Weeks	3325.791	7	475.113	648.752	<.001
Concentration	538.750	8	67.344	91.956	<.001
Species BA/2iP	245.968	3	81.989	111.954	<.001
Weeks * concentration	778.264	56	13.899	18.977	<.001
Weeks * species BA/2iP	1468.206	21	69.915	95.466	<.001
Concentration * species BA/2iP	692.743	24	28.864	39.413	<.001
Weeks*concentration*species BA/2	1745.021	168	10.387	11.933	<.001
Error	632.750	864	0.7323		
Total	9427.492	1151			

Figures 142 to 145.

Mean number of shoots formed per week on intact plants after BA or 2iP treatment.

Fig 142 A. uva-ursi BA.

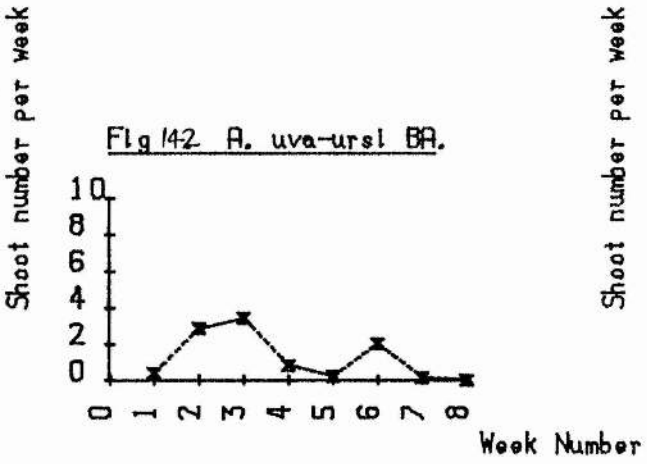


Fig 143 A. uva-ursi 21P.

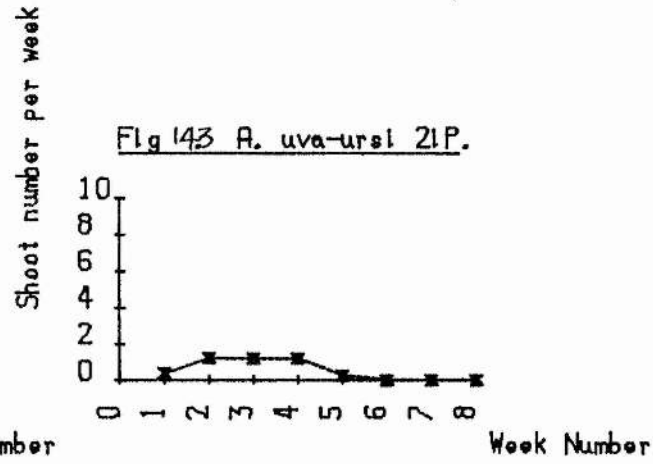


Fig 144 S. 'Froebelii BA.

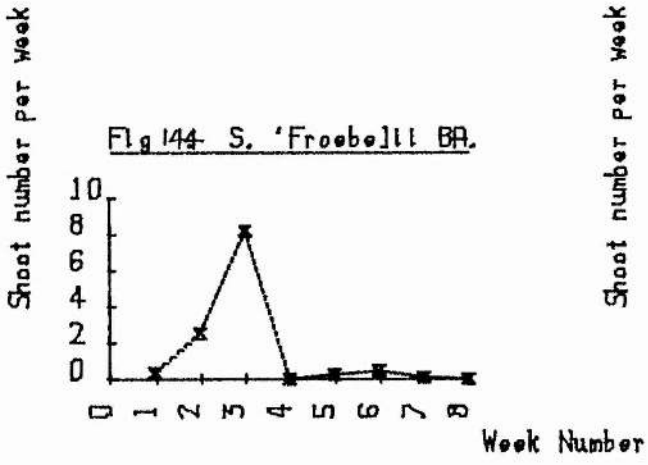


Fig 145 S. 'Froebelii' 21P.

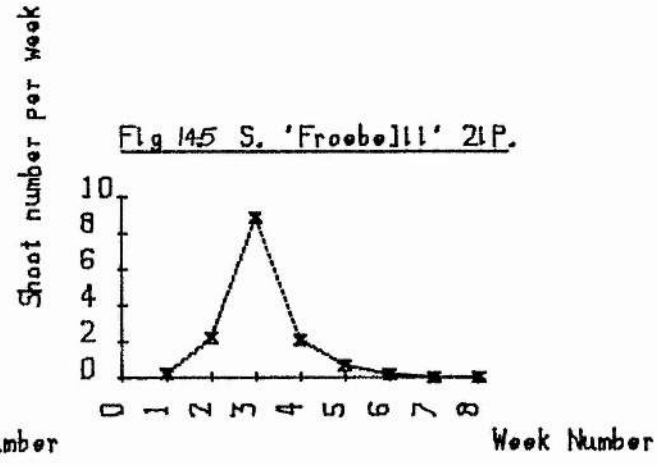


Plate 5.

Plants of Spiraea 'Froebellii' after spray treatment with BA (left) or H_2O (right).

Plate 6.

Axillary shoots on plant of Arctostaphylos uva-ursi after cytokinin treatment.

3.14 DISCUSSION

Shoot formation in vitro

Section 3.11 demonstrated that, in Rosaceae, a greater number of shoots were formed when shoot explants were supplied with BA than when they were treated with 2iP. Similarly, Lundergan and Janick (1980) showed that BA was more effective than 2iP in inducing shoot proliferation in apple. However, shoots were shorter on BA than on 2iP media. This suggests a difference in activity between the two cytokinins.

Variability of plant growth response to different cytokinins has previously been observed; growth rate of tobacco callus varied with cytokinin used (Skoog et al., 1967), and growth characteristics, for example, rate of division, of cell suspensions of soybean reflect differing cytokinin treatments (Everett et al., 1978). Differences in activity have also been reported for excised plant parts, for example, in excised radish cotyledon bioassays (Letham, 1972).

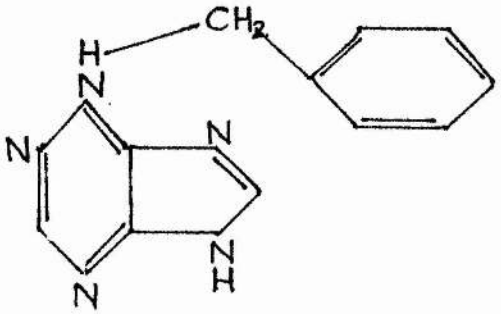
There are several possible explanations for these differences in activity. The activity of growth regulators is believed to be related to their ability to bind to receptor sites within the cell (Letham,

1978), but, for the growth regulator to bind to a given site on a molecule, the growth regulator must possess a particular structure. Investigation of structure and activity relationships have shown associations between structure and activity (Leonard, 1974; Skoog, 1973; Skoog and Armstrong, 1970; Mok, Mok and Armstrong, 1978). The emphasis of these papers has been to study activity in stimulation of cell division in callus.

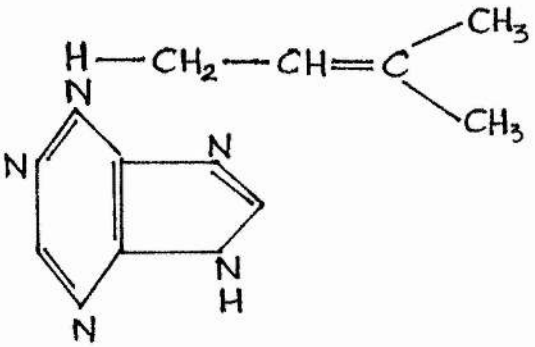
However, Yamada et al. (1972) showed that although both dextrorotatory and levorotatory zeatin compounds induced callus growth in tobacco, only levorotatory compounds promoted shoot formation. This suggests that a high level of activity of cytokinins at one stage of growth (in this case callus growth), does not necessarily indicate that the same compound will have high activity in another growth phase (for example, in differentiation). As a cytokinin may have varying activity in different growth phases, different receptor sites may be involved for each growth phase. Yamada and co-workers suggested that binding of the N_6 group to a receptor site is involved in organogenesis.

Both of the cytokinins studied in the current work have N_6 groups (see below) and both stimulated shoot formation. This supports the hypothesis of Yamada et

al. (1972). However, differences between the two N_6 cytokinins tested were observed with respect to their shoot promoting activity.



BA STRUCTURE



2IP STRUCTURE

The extent to which cytokinins are inactivated in plants may also affect the plant response. From their work on BA activity in soybean cultures, Everett et al (1978) showed that BA forms a mainly inactive conjugate in soybean, and Laloue (1975) reported that in tobacco cell cultures, both BA and 2iP are metabolized to 7-glucosides, the glucosylation process leading to inactivation of the cytokinin. Both of the cytokinins being currently considered display no differences in their metabolism with respect to glucosylation.

Whitty and Hall (1974) purified the enzyme N_6(-isopentenyl)adenosine oxidase from Zea mays. This enzyme degrades naturally occurring cytokinins by cleaving the isopentenyl side chain at the double bond with resultant loss of cytokinin activity. The synthetic cytokinin BA is not reactive but 2iP is one of the most active substrates for the enzyme (Whitty and Hall, 1972).

Exogenous 2iP applied to shoot cultures would then be continuously broken down by the enzyme and thus, a high concentration of 2iP would be necessary for the stimulation of shoot formation. This was the case in the current experiments, where a concentration of 5.0 to 15 mg l^{-1} 2iP was required to stimulate shoot

formation and only 0.1 to 2.5 mg l^{-1}BA (not oxidized) was required to produce the same effect. This is also supported by results which showed that when shoots were cultured for eight weeks, a second growth of shoots formed with BA but not with 2iP suggesting that 2iP had been degraded but that BA had not been inactivated; and by results which showed that a longer exposure period to 2iP was necessary than for BA to produce the same number of shoots.

Other factors may also play a part in controlling cytokinin concentration and thus cytokinin activity. Transport of cytokinin within the explant could affect the activity at a particular site. Ramina et al (1979) showed that BA was transported acropetally in shoots of Phaseolus vulgaris and that the transport rate depended on the light regime. Pilet et al (1967), reported both acropetal and basipetal translocation in excised plant parts. There are no conclusive reports of transport of 2iP in plants and therefore a conclusion cannot be reached on whether differential transport of the two cytokinins tested could account for some of the differences observed in their activity.

The greater activity of BA than 2iP in shoot production may therefore be due to degradation of 2iP

but not of BA by cytokinin oxidase enzyme. However, Ericaceous species formed more shoots on medium containing 2iP than on BA medium. This would seem to contradict the above suggestion. However, BA is toxic to some Rhododendron cultivars (Anderson, 1975) and this was also noted in the current experiments. This may account also for the low shoot number recorded in Ericaceous species. Several Ericaceous species forming few shoots ceased active growth. This was in marked contrast to other Ericaceous species, for example, Arctostaphylos and Vaccinium, which formed numerous shoots and grew rapidly.

Degradation of BA by cleavage of the benzyl group from the N_6 position occurs in some plants (Fox et al., 1972). The enzyme system responsible for this degradation is not known (Letham and Palni, 1983). It is possible, therefore, that Ericaceous species possess this enzyme system but Rosaceous species do not, thus leading to the observed differences in response to BA and 2iP in the plant families.

Shoot number varied with concentration of both BA and 2iP. It was noted that both cytokinins acted over a wide concentration range and this supports the hypothesis that their activity is not controlled by

concentration. However, some species, Potentilla, Spiraea and Vaccinium give a marked response over a narrow concentration range and this may indicate a trigger effect. Therefore, cytokinin concentration may act as a trigger for morphogenesis in some species (see discussion below).

Shoots formed more rapidly when cytokinin concentration was high than at low concentrations. This suggests that there may not be many receptors for cytokinin present in the explant initially, and that at low concentrations, cytokinin molecules do not readily chance on these receptors, whereas at high concentrations, there is a greater likelihood of binding with a large proportion of receptor molecules. If this is so, these results may indicate that, in order for cytokinins to be active at low concentrations, the cytokinin could promote synthesis of receptor molecules as has been shown for the animal hormone oestradiol (Cohen and Hamilton, 1975).

Longer shoots were formed in 2iP treatments than in BA treatments. This may indicate 1) that shoots formed on 2iP medium formed earlier, 2) that an interaction of cytokinins with either auxins or gibberellins (cell elongation promoters) occurs or 3)

that 2iP itself promotes and BA inhibits shoot elongation.

Experiments to determine rate of shoot formation showed that shoots formed earlier on medium containing BA. Therefore hypothesis (i) above was invalidated.

Other workers have shown a reduction in elongation of shoots by cytokinin. Cytokinins, including BA and 2iP, reduced shoot extension and needle length in Pinus; cytokinins inhibited the elongation of isolated stem segments of Raphanus hypocotyls (Letham, 1971) and of Pisum (Sasse et al., 1972); and the formation of stunted shoots on media containing BA has been reported in apple at 3 to 5 mg l^{-1} (Lundergan and Janick, 1980; Lundergan and Janick, 1979) and at 10 mg l^{-1} (Jones, 1967). Jones (1967) and Elliot (1972) reported that BA at low concentrations promoted shoot elongation. Skoog and Abdul Ghani (1981) showed that 2iP was more active than BA in stimulating lateral bud growth in Pisum.

Einset (1977) has shown that 2iP modified auxin requirement and auxin production in tobacco callus cultures. Lee (1971) reported that exogenous cytokinin in tobacco tissue cultures affects the distribution of indole acetic acid oxidase isozymes, and cytokinins have been shown to inhibit the auxin response of

isolated stem segments in Pisum (Brian and Hemming, 1957) and cucumber hypocotyl (Katsumi and Kazama, 1974). Therefore, the promotion of shoot elongation by 2iP could be due to an indirect effect of auxin action. However, BA has also been shown to cause an increase in the level of free IAA (Imaseki et al., 1975). It is possible that BA promotes the synthesis of quantities of IAA which are inhibitory to growth. This possibility is supported by data from my experiments which showed a decrease in shoot length with increasing BA concentration. However, Imaseki et al. (1975) reported only a slight increase in the level of IAA after BA treatment and so the presence of inhibitory levels of IAA in the shoot is unlikely.

BA enhanced ethene synthesis in mung bean hypocotyls (Imaseki et al., 1975) and this was also shown in Chapter 8 of this thesis. The presence of high ethene levels could lead to inhibition of shoot elongation and promotion of cell expansion. In the current experiments, BA-cultured shoots were observed to have swollen stems and this supports the hypothesis that ethene could be responsible for the stunting of shoots cultured on medium containing BA.

Total shoot length was similar on BA and 2iP

medium - BA giving many short shoots and 2iP giving few longer shoots. Growth of the initial explant was recorded to test whether its growth was greater on BA than on 2iP medium and therefore whether greater apical dominance was evident in explants grown on BA medium. It was shown that initial explant growth was less with BA than with 2iP. It can be concluded that apical dominance is unlikely to be responsible for the observed difference in shoot length.

The results of Section 3.12 indicate that there is a restricted number of sites from which shoots can originate in the shoot explants used. Most shoots were formed in the first three weeks of culture. However, on BA media, additional shoots formed in the fifth and sixth weeks. Most of these were secondary shoots i.e. branches on the shoots which had formed earlier. This implies that BA was not inactivated although shoot initiation ceased. The fact that more shoots formed later suggests that all suitable sites for shoot initiation had been filled by the fourth week of culture and that more shoots could only form if additional sites became available i.e. when primary shoots had grown sufficiently for sites to be available.

Secondary shoots were axillary shoots formed from axillary shoots but the species in which secondary shoots formed were shown to form adventitious shoots as well as axillary shoots. These species then, are able to form shoots de novo. Thus, it would be expected that there would be no limit to the number of shoots which could form if nutrient, energy and growth regulator requirements are met. It may be that the shoot explant can only support a limited number of shoots due to competition between shoots , so that although sufficient of these requirements may be present in the medium, the rate of absorption limits the number of shoots which one explant can support.

An alternative explanation could be that BA in the medium is exhausted after the first four weeks but that the shoots are capable of synthesising more cytokinin which leads to shoot formation. This would not however explain why the new shoots were formed on primary shoots rather than on the original shoot explant, nor why a second flush of shoots was formed in BA treatments but not in 2iP treatments. Also, cytokinins are normally synthesised in roots, although cytokinin synthesis can occur in callus and other isolated tissues (Chen and Melitz, 1979). The hypothesis of a

limited number of sites being available therefore seems most probable.

The shoots formed were either axillary or adventitious in origin. The shoot origin varied with species. Adventitious shoots were formed in only 6 of the 20 Ericaceous species and cultivars tested, but in 8 of the 10 Rosaceous species and cultivars. All species formed axillary shoots. A difference was also noted between BA and 2iP treated shoots - in Rosaceae, 8 species and cultivars formed adventitious shoots on BA medium, whereas only 4 cultivars formed adventitious shoots when treated with 2iP.

Other workers have reported the simultaneous formation of axillary and adventitious shoots in Rosaceae (Abbott and Whiteley, 1976; Jones et al., 1979) and in Ericaceae (Anderson, 1978a); and Nickerson (1978) reported the formation of adventitious shoots in Vaccinium and Anderson (1975) reported the formation of axillary shoots but no adventitious shoots in Rhododendron.

Murashige (1977c) used different media to stimulate either axillary or adventitious shoot formation. His shoot multiplication medium 'A' containing 30 mg l^{-1} 2iP and 0.3 mg l^{-1} IAA stimulated

axillary branching, whereas shoot multiplication medium 'B' containing 2 mg l^{-1} kinetin and 2 mg l^{-1} IAA induced formation of adventitious shoots. A much higher cytokinin / auxin ratio was therefore necessary for axillary shoot formation. This was not observed in the experiments reported in this thesis as both adventitious and axillary shoots were formed at the same concentration.

Murashige (1977c) used two different cytokinins to promote the two different types of shoot formation. Although he has not stated it to be the case, this may be because 2iP does not promote adventitious shoot formation in many species. Murashige used kinetin to induce adventitious shoot formation and my experiments showed that BA was more active in promotion of adventitious shoots than was 2iP. BA and kinetin are both synthetic cytokinins and therefore, it is possible that there is a difference in mode of action between synthetic and natural cytokinins in shoot initiation. As discussed above, this difference could be due to degradation of natural cytokinins by cytokinin oxidase.

It was also observed that the effective concentration range for adventitious shoot frormation was less than for axillary shoot formation. Therefore,

concentration is more critical for de novo organ formation and may be the controlling factor whereas axillary shoot formation may not be controlled by cytokinin concentration. This was particularly notable in Potentilla when BA was supplied (Figures 5 and 6). The peak in shoot number at low BA concentration represents the formation of a small number of axillary shoots and a larger number of adventitious shoots. The sudden decrease in shoot number with increase in BA concentration is due to the failure to form adventitious shoots, and shoot number at high BA concentrations is entirely due to axillary shoot formation.

Shoot formation in intact plants

Shoots formed on intact plants as a result of cytokinin spray treatment, were, in all cases, axillary in origin. This factor may account for the low shoot number recorded in intact plants.

This experiment showed no adventitious shoot formation in intact plants although three of the species tested had been shown to form adventitious shoots in vitro. Environmental conditions (temperature and light) were identical to those

provided for in vitro experiments and a range of cytokinin concentrations were supplied. Therefore, formation of adventitious shoots is most likely to be due to 1) the excision process or 2) additional mineral or carbohydrate availability. These are discussed further in Chapters 9 and 6 respectively.

3.2 THE ROLE OF ENDOGENOUS FACTORS IN SHOOT FORMATION

Section 3.1 demonstrated the importance of cytokinin in shoot initiation. However, differences in exogenous cytokinin concentration requirement were observed between species. Endogenous factors, for example, auxin or other growth regulators, may account in part for this variability in response.

Endogenous growth regulator content is known to vary from one part of the plant to another and with season. Thus, a study of shoot initiation in different plant parts and in different seasons will show the importance of endogenous factors in determining shoot initiation. The following factors were selected for experimentation :- explant size, presence of axillary buds, presence of shoot apex, maternal derivation of explant and season of experimentation.

Addition of a range of concentration of cytokinin to media will also highlight the importance of factors other than cytokinin in shoot formation.

3.21 EXPLANT FACTORS

Method

The species used for the following experiments were *Prunus cerasifera* and *Spiraea* 'Froebelii'. BA was incorporated in the nutrient medium at 0.1, 0.5 or 1.0 mg 1-1. Incubation was in light (16 hour photoperiod) as previously specified.

Experiment 1 - Explant size

Shoot tips 2.5, 5.0, 7.5, 10.0, 15.0 and 20 mm were cultured. Shoot number and length, and explant length were recorded at the end of a four week culture period.

Experiment 2 - Presence of axillary buds

Explants were prepared with 0, 1, 2, 3, 4, 5 or 6 axillary buds. Buds were removed from explants to obtain these bud numbers.

Shoot number and length and explant length were recorded at the end of a four week culture period.

Experiment 3 - Presence of shoot apex

The terminal three millimetres of the shoot tip was either removed or left intact. Shoot number and length and explant length were recorded at the end of a four week culture period.

Experiment 4 - Derivation of explant

Shoot explants were selected from the top, middle and base of the plant. Shoot number and length and explant length were recorded at the end of a four week culture period.

Results

Experiment 1 - Explant size

Number of shoots increased with length of the initial explant in both species and at all BA concentrations $p<.001$)(Figures 146 and 147). Shoots 5mm or less formed disproportionately fewer shoots than longer shoots. Length of new shoots did not vary with original explant length when explants were 7.5 mm or longer, but was reduced when the original explant was less than 7.5 mm(Figures 148 to 153). The initial explant increased in length to a greater extent in long

explants (Figures 154 to 159).

Experiment 2 - Presence of axillary buds

Number of shoots formed decreased with number of buds present (Figures 160 and 161). This decrease was significant ($p<.001$) at all concentrations tested. Length of shoots (Figures 162 to 167) and growth of initial shoot explant (Figures 168 to 173) also decreased with increasing bud number.

Experiment 3 - Presence of shoot apex

At a BA concentration of 0.1 mg l^{-1}, a significantly greater number of shoots was formed when the apex had been excised (Table 48). However, at higher BA concentrations, there was no significant difference between shoot number in the presence and absence of the apex. An analysis of variance was conducted (Table 49) and this showed a significant difference due to presence of apex ($p<.001$), BA concentration ($p<.001$), species ($p<.001$) and a significant interaction between these ($p<.01$ - $p<.001$).

Modal shoot length was increased when the apex was absent in both species and at all BA concentrations (Figures 174 and 175). Extension growth of the initial explant was inhibited by removal of the shoot apex

(Table 50).

Experiment 4 - Derivation of explant

A significant difference ($p<.001$) in shoot number between explants from the top, middle and base of the parent plant was recorded at all BA concentrations in Spiraea, and between top and base explants in Prunus at 0.5 mg l^{-1} BA. (Table 51). Explants taken from the top of the parent plant formed the greater number of shoots. A decrease was also observed at 0.1 and at 1.0 mg l^{-1} BA in Prunus but this difference was not significant at the 1% level (Table 51). An analysis of variance demonstrated a significant effect ($p<.001$) due to presence of the apex, BA concentration and species, and a significant interaction ($p<.001$) between these (Table 52).

No difference in modal shoot length was recorded at a BA concentration of 1.0 mg l^{-1} (Figures 178 and 181). However, at lower BA concentrations, shoots formed on explants taken from the top of the parent plant were longer than shoots formed on explants taken from the base (Figures 176, 177, 179, 180). Initial explant growth was less in basal explants in Prunus (Figures 182 to 184) and in Spiraea at 0.1 mg l^{-1} BA (Figure 185). However, no difference in explant growth

was found in Spiraea when cultured at 0.5 or 1.0 mg l^{-1} BA(Figures 186 to 187).

Figures 146 and 147.
Mean shoot number at the end of a four week culture period on medium containing BA : change with explant length.

Figures 148 to 153.
Modal shoot length at the end of a four week incubation period on medium containing BA : change with initial explant length.

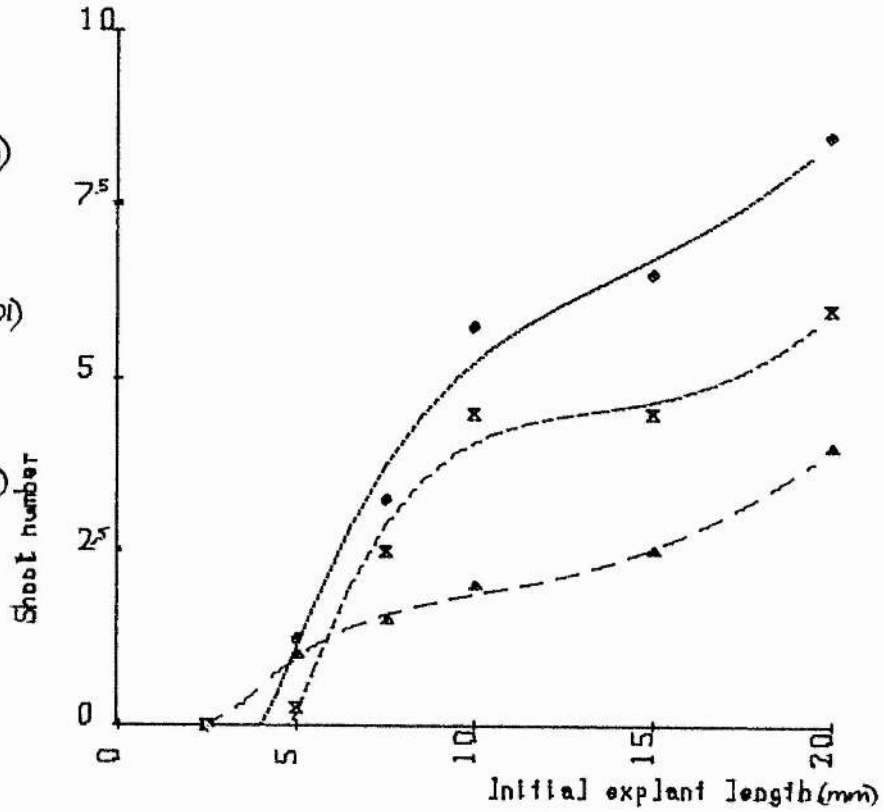
Figures 154 to 159.
Explant growth in a four week incubation period on medium containing BA : change with initial explant length.

Fig 146 Prunus cerasifera

0·1 mg l^{-1} BA
R^2 = ·9882
SE = ·5967
r = ·9941 (p<·001)

0·5 mg l^{-1} BA
R^2 = ·9890
SE = ·7666
r = ·9940 (p<·001)

1·0 mg l^{-1} BA
R^2 = ·9972
SE = ·1608
r = ·9986 (p<·001)



x 0·1 mg l^{-1} BA

◆ 0·5 mg l^{-1} BA

▲ 1·0 mg l^{-1} BA

Fig 147 Spiraea 'Froebelii'.

0·1 mg l^{-1} BA
R^2 = ·9940
SE = 1·844
r = ·9970 (p<·001)

0·5 mg l^{-1} BA
R^2 = ·9991
SE = ·9012
r = ·9996 (p<·001)

1·0 mg l^{-1} BA
R^2 = ·9895
SE = 3·419
r = ·9947 (p<·001)

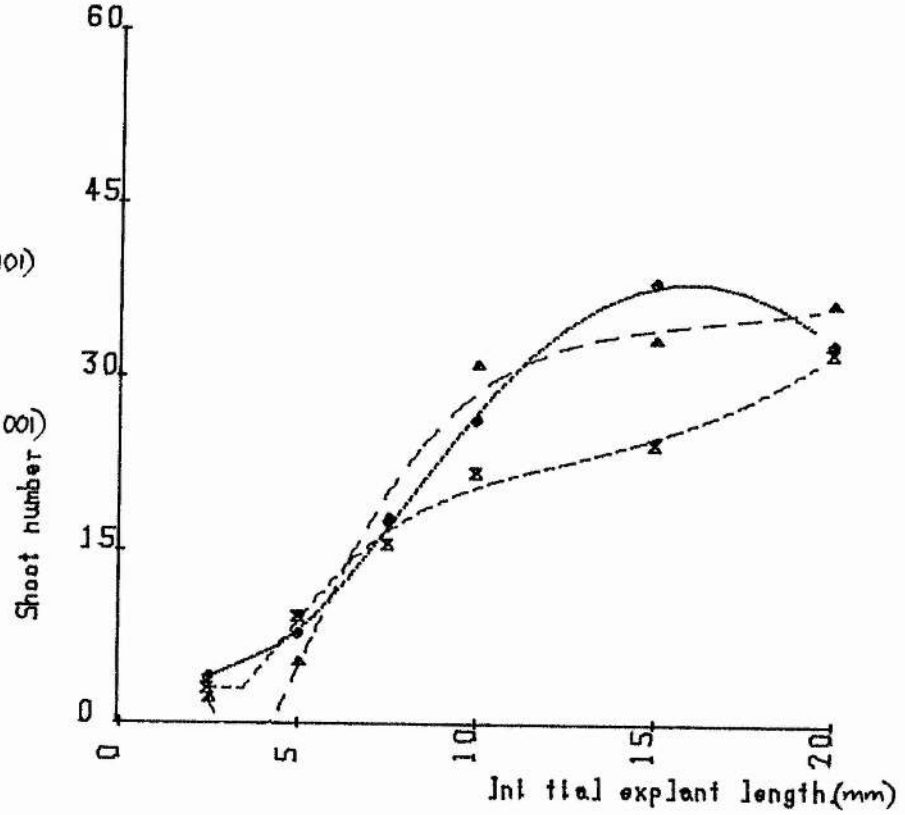


Fig 148. Spiraea 'Froebelii' 0.1mg l^{-1} BA

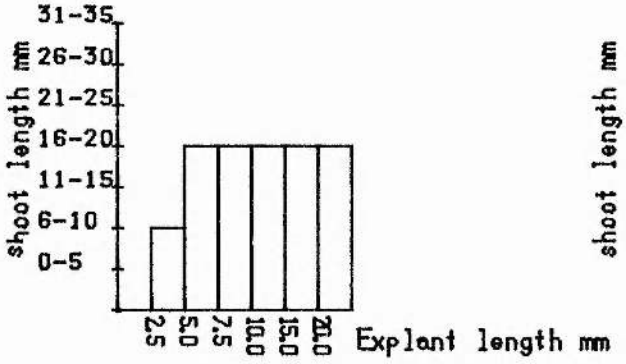


Fig 149. Spiraea 'Froebelii' 0.5mg l^{-1} BA

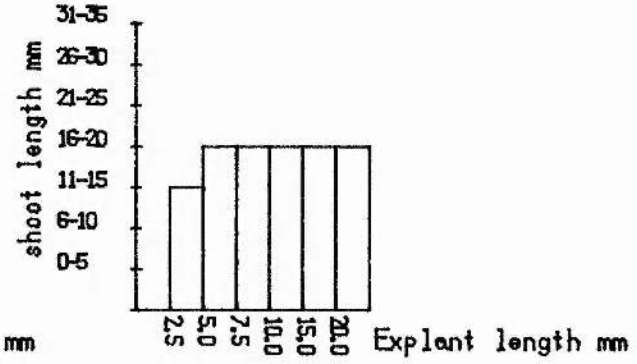


Fig 150. Spiraea 'Froebelii' 1.0mg l^{-1} BA

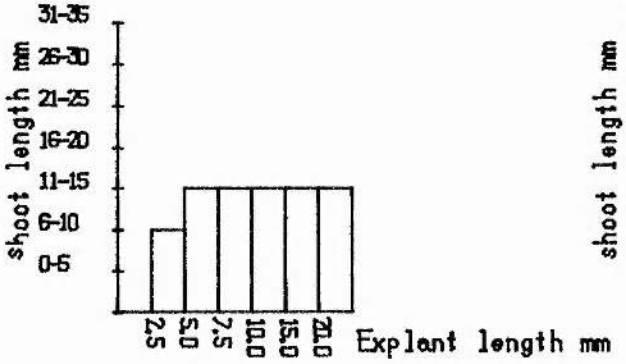


Fig 151. Prunus cerasifera 0.1mg l^{-1} BA

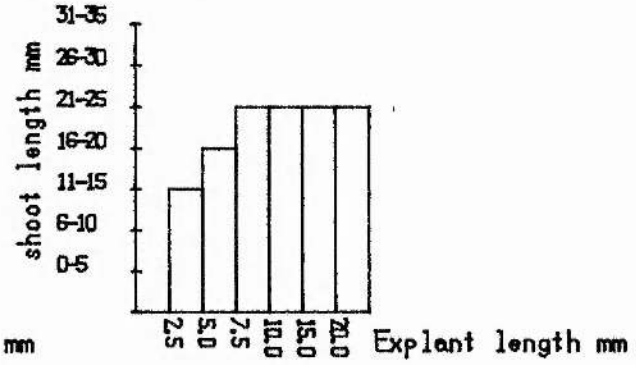


Fig 152. Prunus cerasifera 0.5mg l^{-1} BA

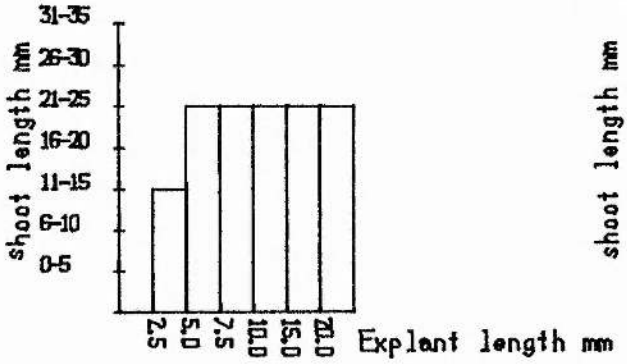


Fig 153. Prunus cerasifera 1.0mg l^{-1} BA

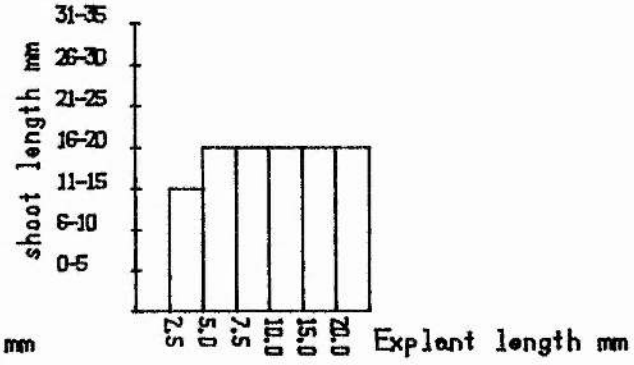


Fig 154. Spiraea 0.1mg l^{-1}BA

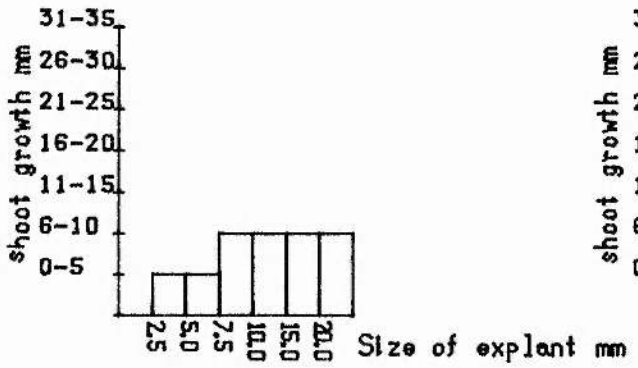


Fig 155. Spiraea 0.5mg l^{-1}BA

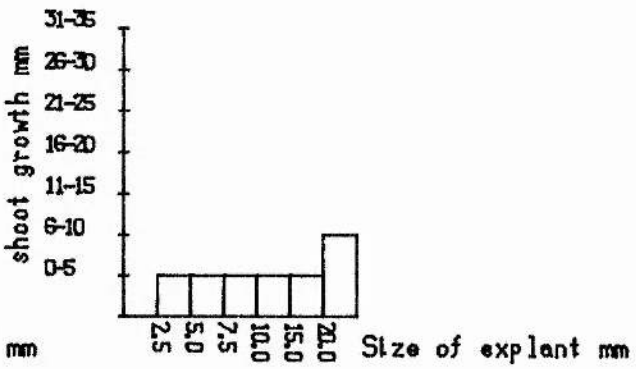


Fig 156. Spiraea 1.0mg l^{-1}BA

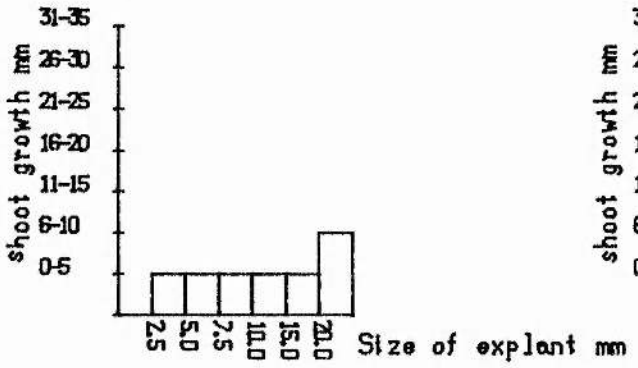


Fig 157. Prunus 0.1mg l^{-1}BA

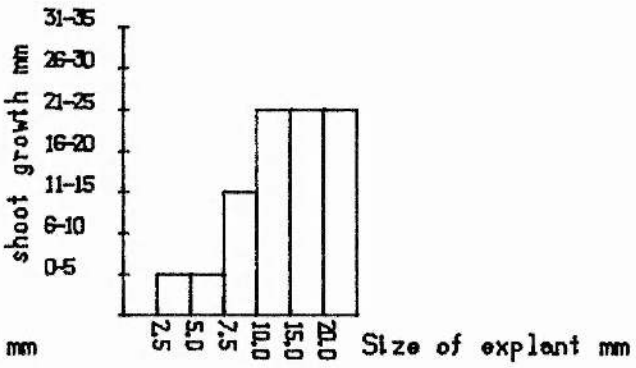


Fig 158. Prunus 0.5mg l^{-1}BA

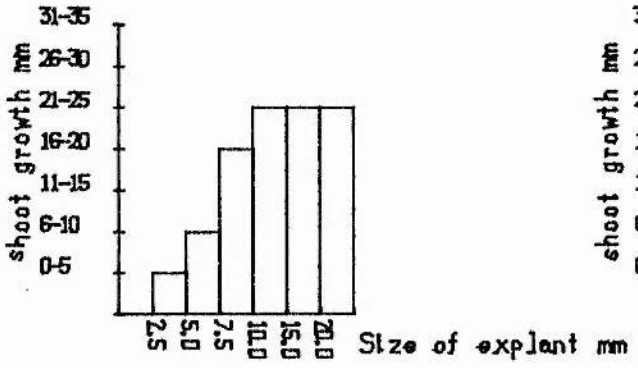


Fig 159. Prunus 1.0mg l^{-1}BA

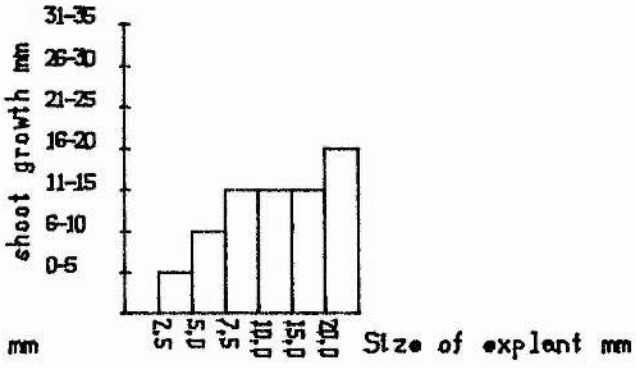
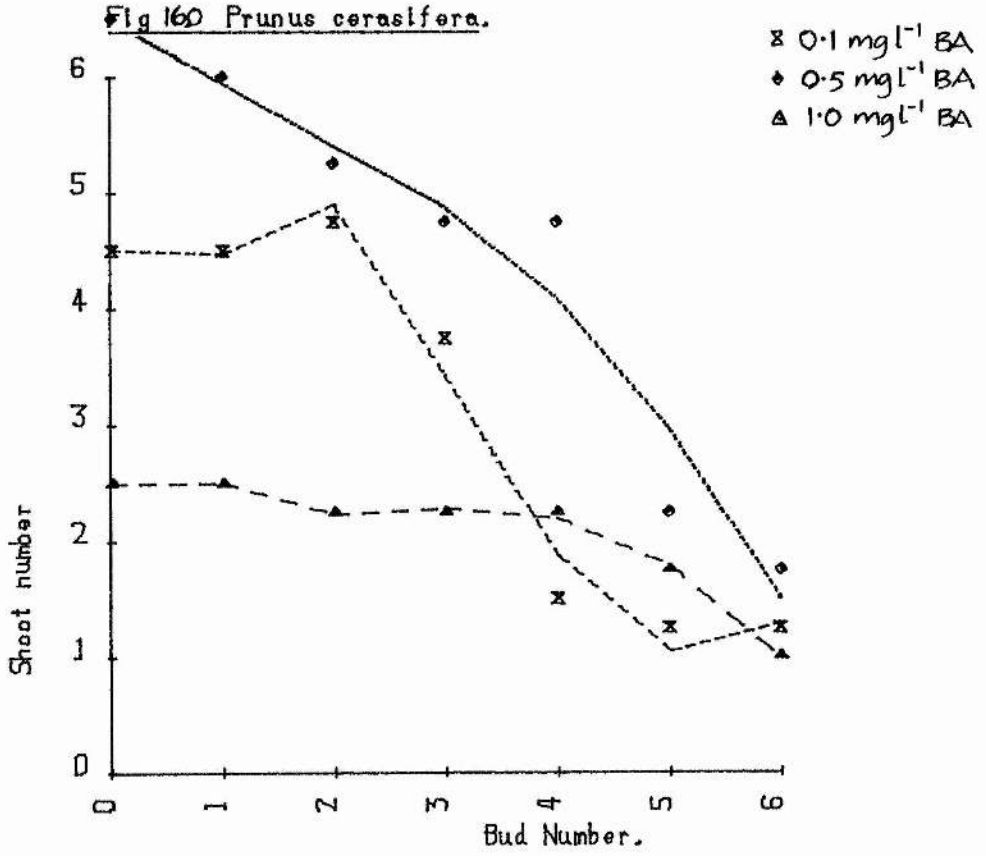


Fig 160 Prunus cerasifera.



Figures 160 and 161.
Mean shoot number at the end of a four week incubation period on medium containing BA : change with bud number.

Figures 162 to 167.
Modal shoot length at the end of a four week incubation period on medium containing BA : effect of bud number.

Figures 168 to 173.
Explant growth in a four week incubation period on medium containing BA : effect of bud number.

Fig 161. Spiraea 'Froebelii'.

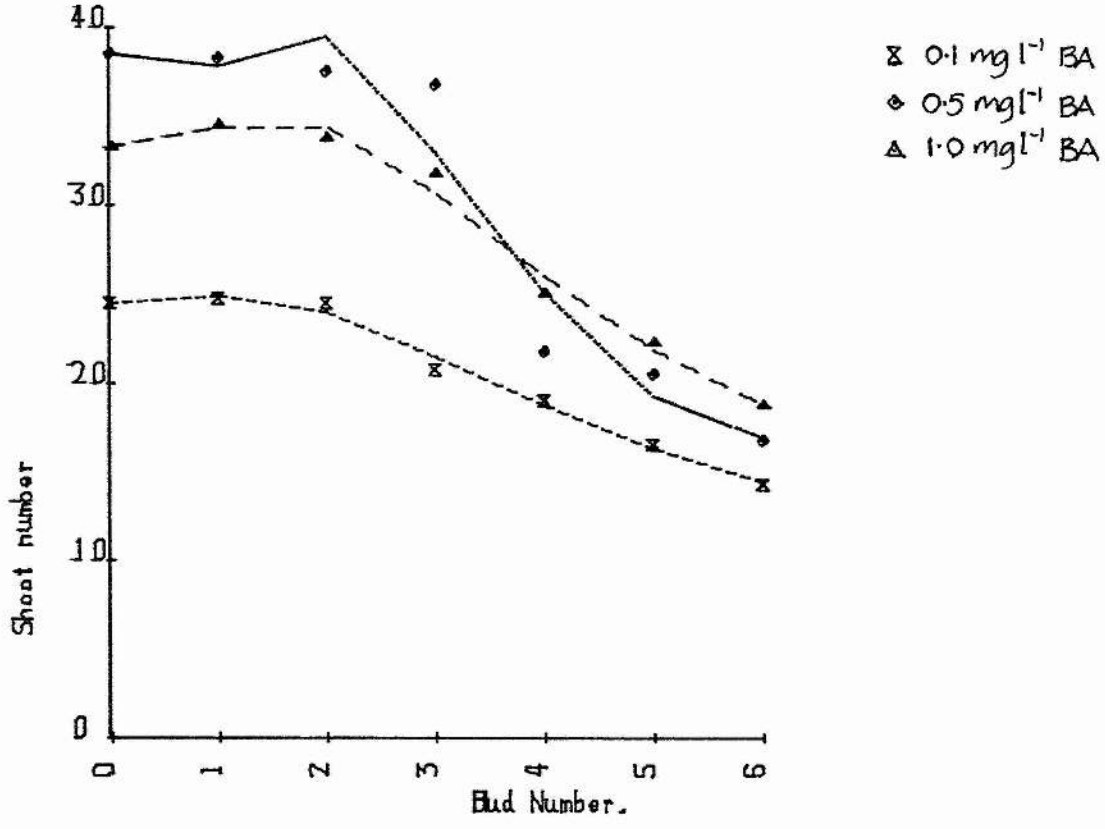


Fig 162. Spiraea 0.1mg l^{-1}BA

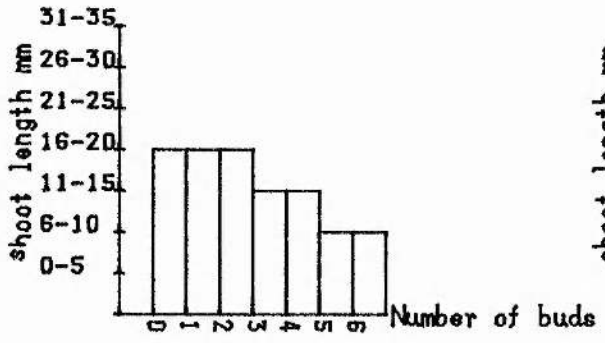


Fig 163. Spiraea 0.5mg l^{-1}BA

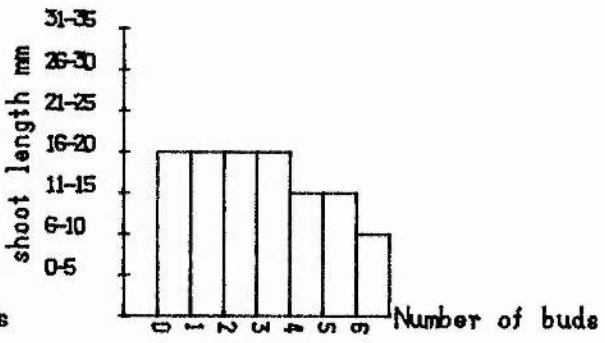


Fig 164. Spiraea 1.0mg l^{-1}BA

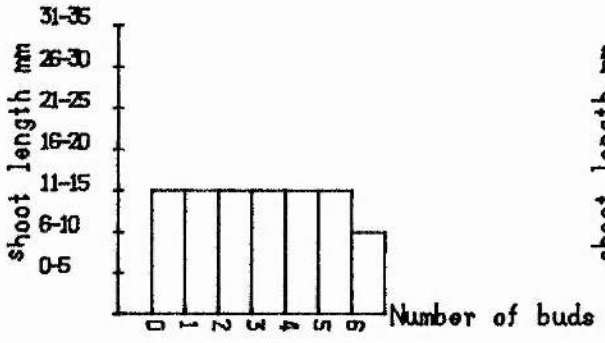


Fig 165. Prunus 0.1mg l^{-1}BA

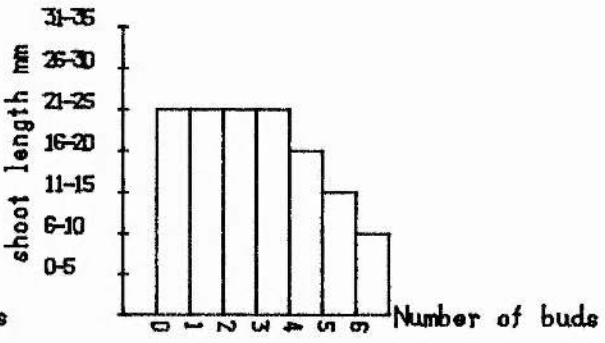


Fig 166. Prunus 0.5mg l^{-1}BA

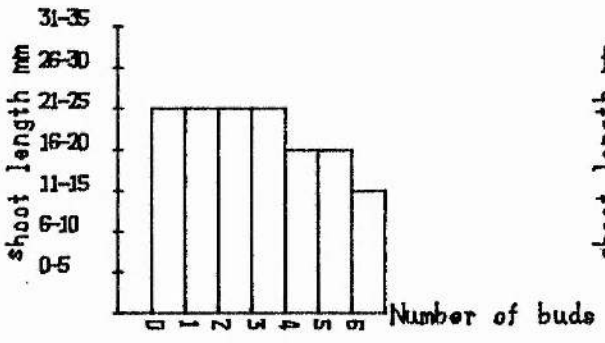


Fig 167. Prunus 1.0mg l^{-1}BA

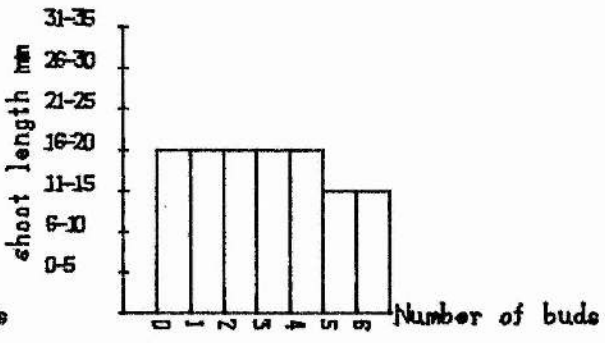


Fig 168. Spiraea 0.1mg l^{-1}BA

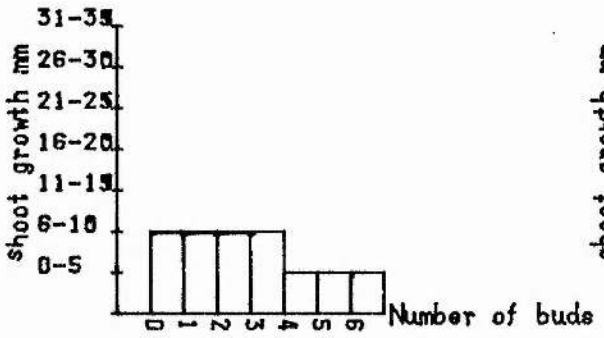


Fig 169. Spiraea 0.5mg l^{-1}BA

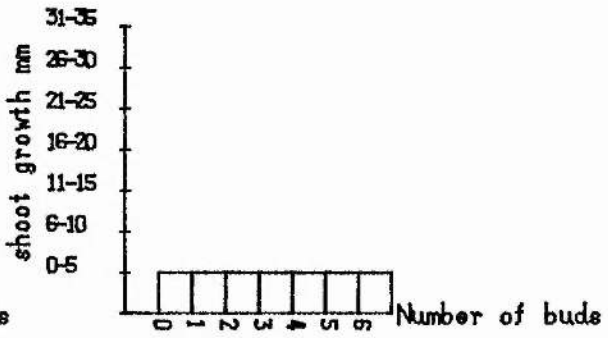


Fig 170. Spiraea 1.0mg l^{-1}BA

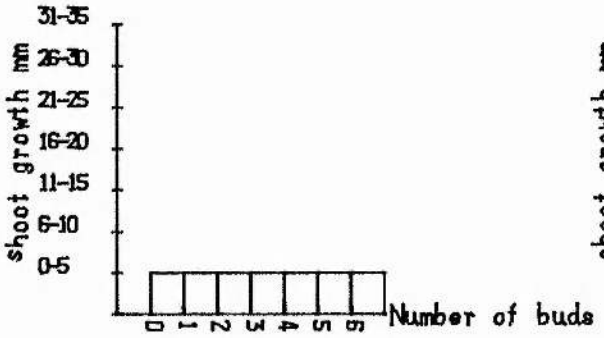


Fig 171. Prunus 0.1mg l^{-1}BA

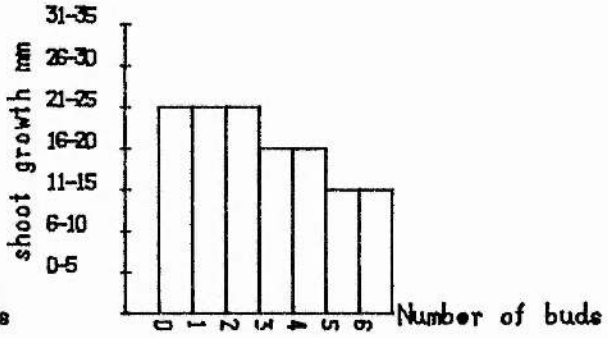


Fig 172. Prunus 0.5mg l^{-1}BA

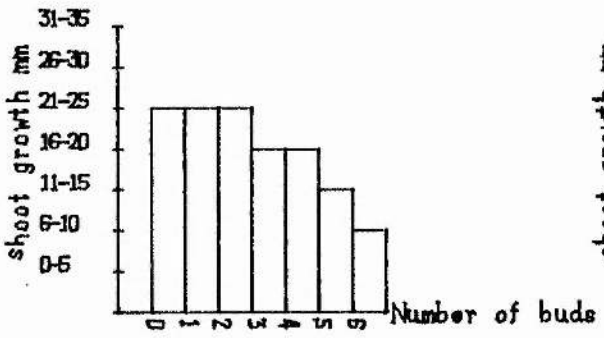


Fig 173. Prunus 1.0mg l^{-1}BA

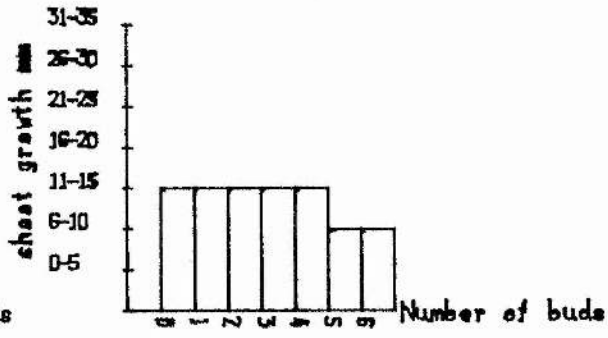


Table 48.

Mean shoot number after BA treatment in presence or absence of apex.

Concentration mg l^{-1} BA	+ apex	- apex
Spiraea 'Froebelii'		
0.1	24.25	31.75
0.5	38.25	39.50
1.0	33.25	33.00
Prunus cerasifera.		
0.1	4.50	6.75
0.5	6.50	7.00
1.0	2.50	3.00

L.S.D. = 1.52 ($p<.05$)

Table 49.

Analysis of variance for data presented in Table 48.

SOURCE	S.S.	D.F.	M.S.	F	p
apex presence	46.021	1	46.021	41.679	<.001
Concentration of BA	325.500	2	162.755	147.396	<.001
Species	9605.021	1	9605.021	8698.887	<.001
Apex * concentration	52.167	2	26.083	23.623	<.001
apex * species	9.187	1	9.187	8.321	<.01
Concentration * species	216.167	2	108.083	97.887	<.001
Apex * concentration * species	19.500	2	9.750	4.160	<.05
Error	39.750	36	1.104		
Total	10313.313	47			

Table 50.

Modal (mm) initial explant growth in a 4 week culture period on medium containing BA : presence or absence of shoot apex.

Concentration mg l^{-1} BA	+ apex	- apex
Spiraea 'Froebelii'.		
0.1	8	0
0.5	3	0
1.0	3	0
Prunus cerasifera.		
0.1	23	0
0.5	23	0
1.0	13	0

Figures 174 and 175.
Modal shoot length at the end of a four week incubation period on medium containing BA : effect of the shoot apex.

Figures 176 to 181.
Modal shoot length at the end of a four week incubation period on medium containing BA : effect of explant derivation.

Figures 182 to 187.
Initial explant growth in a four week incubation period on medium containing BA : effect of explant derivation.

Fig 174. Spiraea 'Froebelii'.

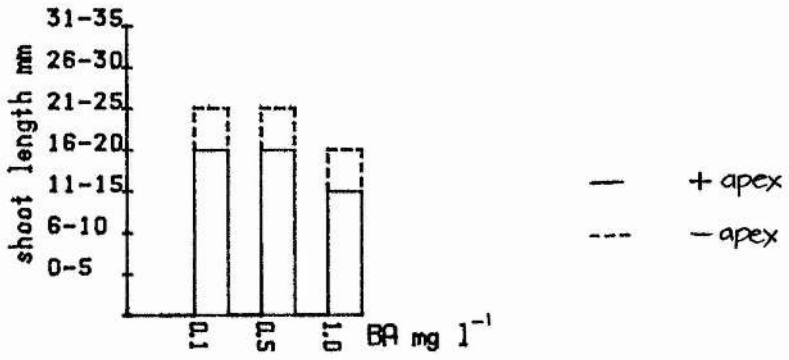


Fig 175. Prunus cerasifera.

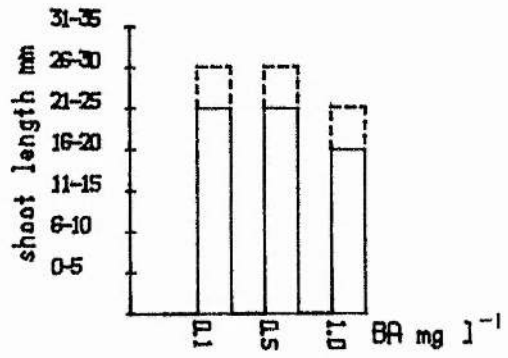


Fig 176. Prunus 0.1 mg l^{-1} BA.

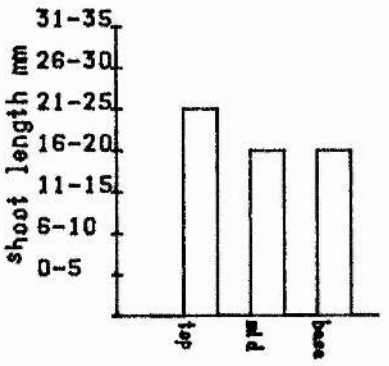


Fig 177. Prunus 0.5 mg l^{-1} BA.

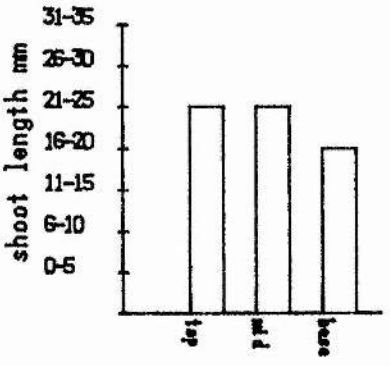


Fig 178. Prunus 1.0 mg l^{-1} BA.

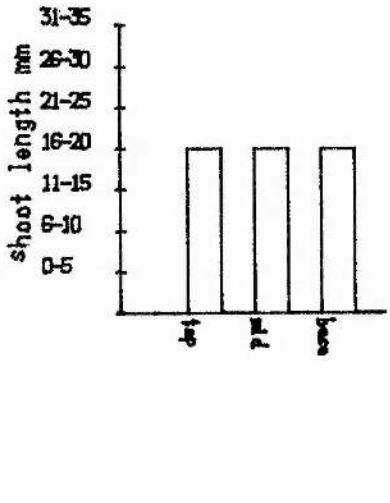
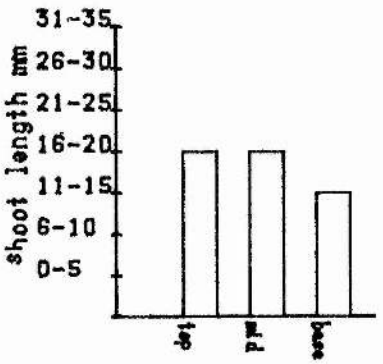
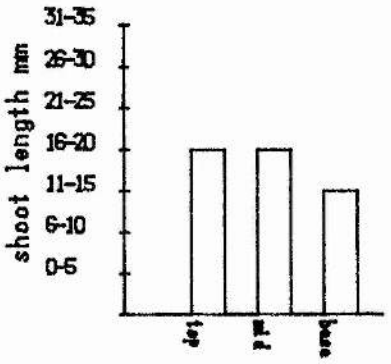


Fig 179. Spiraea 0.1 mg l^{-1} BA.



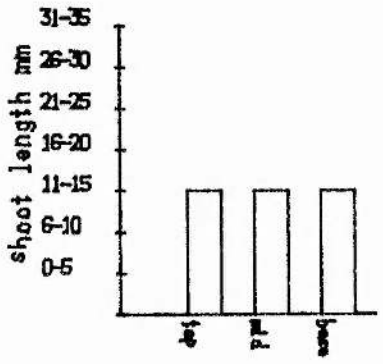
Explant derivation

Fig 180. Spiraea 0.5 mg l^{-1} BA.



Explant derivation

Fig 181. Spiraea 1.0 mg l^{-1} BA.



Explant derivation

Fig 182. Prunus 0.1 mg l^{-1} BA.

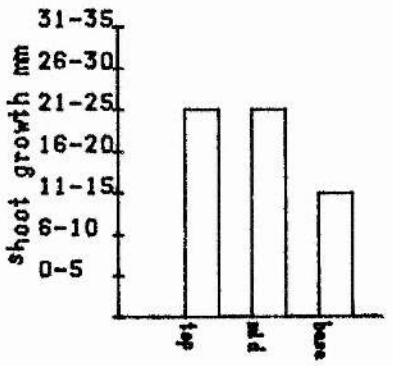


Fig 183. Prunus 0.5 mg l^{-1} BA.

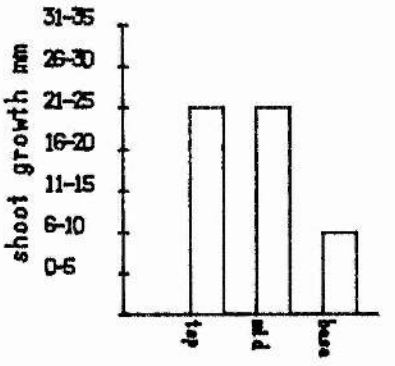


Fig 184. Prunus 1.0 mg l^{-1} BA.

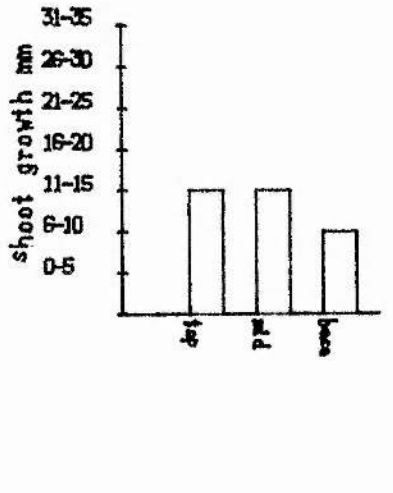
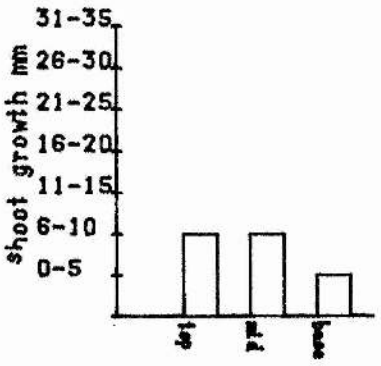
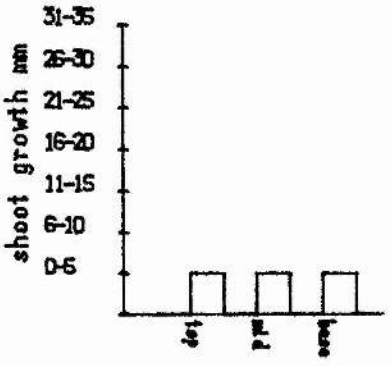


Fig 185. Spiraea 0.1 mg l^{-1} BA.



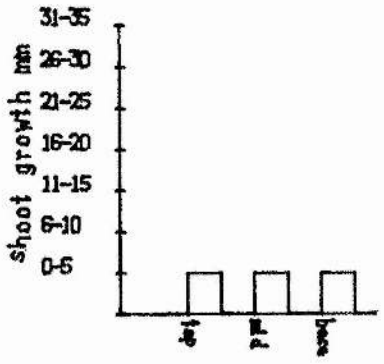
Explant derivation

Fig 186. Spiraea 0.5 mg l^{-1} BA.



Explant derivation

Fig 187. Spiraea 1.0 mg l^{-1} BA.



Explant derivation

Table 51.

Mean shoot number after a four week incubation period on medium containing BA : derivation of explant.

Concentration mg l^{-1} BA	top	middle	bottom
Spiraea 'Froebelii'.			
0.1	24.25	20.50	18.00
0.5	38.25	32.75	22.50
1.0	33.25	28.00	25.25
Prunus cerasifera.			
0.1	4.50	3.50	3.25
0.5	6.50	4.75	4.00
1.0	2.50	2.50	2.00

L.S.D. = 2.29 ($p<.05$) for any two values.

Table 52.

Analysis of variance for data presented in Table 51.

SOURCE	S.S.	D.F.	M.S.	F	p
top/mid/bottom	391.028	2	195.514	77.488	<.001
Concentration of BA	404.528	2	202.264	80.163	<.001
Species	9730.125	1	9730.125	3856.343	<.001
top/mid/bottom * concentration	77.889	4	19.472	7.717	<.001
top/mid/bottom * species	221.583	2	110.792	43.910	<.001
Concentration * species	333.583	2	166.792	66.104	<.001
top/mid/bottom *concentration*speci	49.333	4	12.333	21.955	<.001
Error	136.250	54	2.523		
Total	11344.319	71			

3.22 SEASONAL VARIATION

Method

Experiment 1.

Shoot explants of Prunus cerasifera and Spiraea 'Froebelii' were cultured on nutrient medium containing BA at 0.1, 0.5 or 1.0 mg l^{-1}. Shoots were cultured at monthly intervals during the spring, summer and autumn.

Shoot number and length and explant length were recorded at the end of a four week culture period in light (16 hour photoperiod).

Experiment 2.

To test whether the effect of season on shoot morphogenesis was retained in culture, shoot explants of Spiraea 'Froebelii' initially cultured in August were subcultured to medium containing BA at 0.1, 0.5 or 1.0 mg l^{-1}. Shoot number was recorded at the end of a four week incubation period.

Results

Experiment 1.

Mean shoot number changed with month of culture in both species and at all BA concentrations (Figures 188 and 189). A significant difference ($p<.001$) in shoot number between seasons was demonstrated (Table 53). Shoot number peaked in May and June, declined in July and August, rose again slightly in September, and declined in October. An analysis of variance (Table 54) showed a significant effect ($p<.001$) due to month of culture, BA concentration and species, and a significant interaction ($p<.001$) between these. In Spiraea a BA concentration of 1.0 mg l^{-1} gave most shoots in March, April, July,), August and October. In May, June and September 0.5 mg l^{-1} was the optimal BA concentration for shoot formation. In Prunus, 0.5 mg l^{-1} BA gave most shoots in all months except March and October when 1.0 mg l^{-1} BA was optimal.

Modal shoot length did not change between May and September, but was reduced in March, April and October (Figures 190 to 195). Initial explant growth followed the same pattern in Prunus (Figures 196 to 199), but in Spiraea a slight increase in explant

growth was observed only in May and June. (Figures 200 to 201).

Experiment 2.

A considerable increase in shoot number occurred when August-cultured explants were subcultured (Table 55). This increase was significantly greater ($p<.05$) than in May-cultured explants (Table 56).

Figures 188 and 189.

Mean shoot number at the end of a four week incubation period on medium containing BA : change with season.

Correlation coefficient based on quartic polynomial fit :-

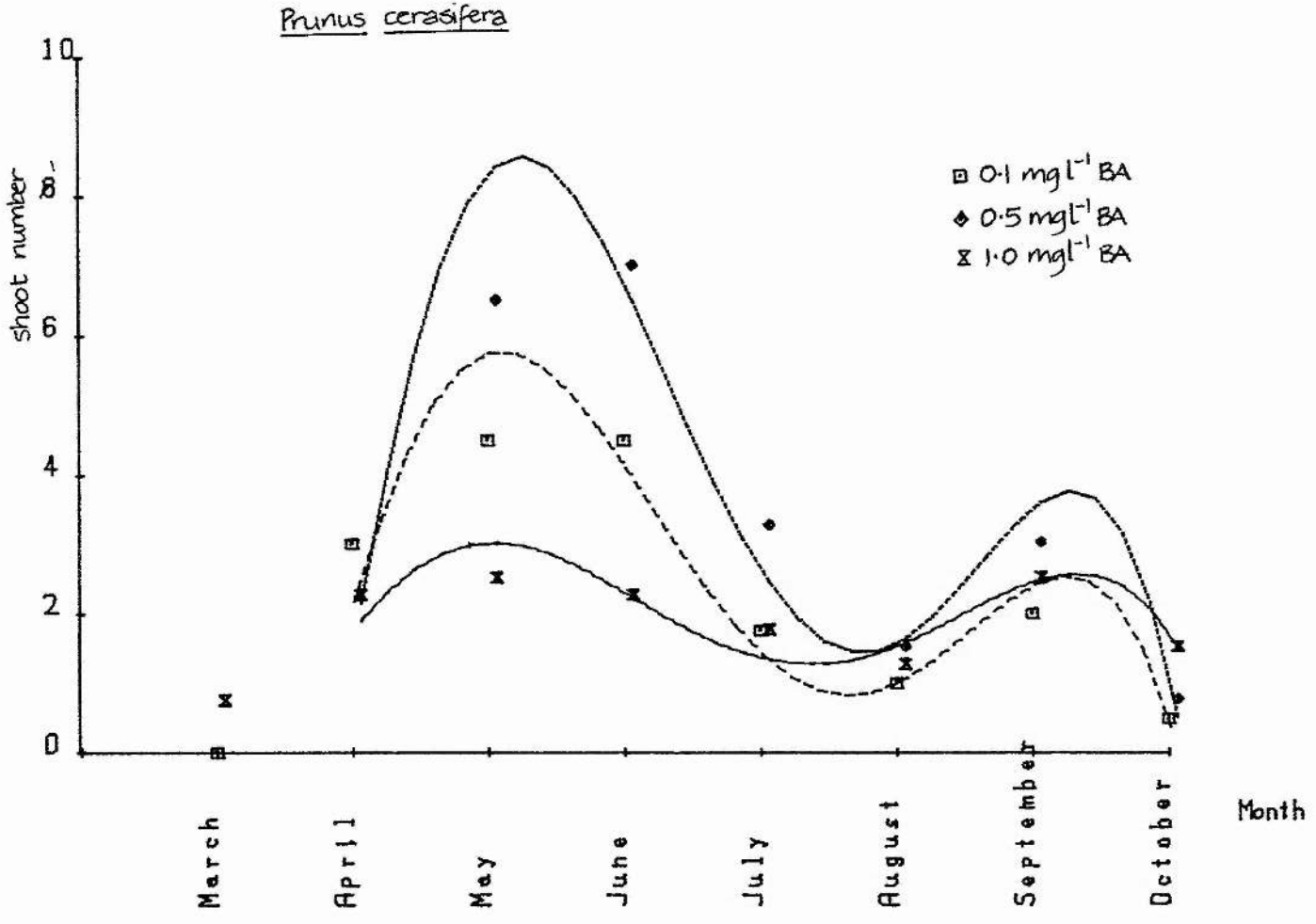
Prunus cerasifera	0.1 mg l^{-1} BA	r = .9292	(p<.001)
	0.5 mg l^{-1} BA	r = .9147	(p<.01)
	1.0 mg l^{-1} BA	r = .8907	(p<.01)
Spiraea 'Froebelii'	0.1 mg l^{-1} BA	r + .9612	(p<.001)
	0.5 mg l^{-1} BA	r = .9677	(p<.001)
	1.0 mg l^{-1} BA	r = .9890	(p<.001)

Figures 190 to 195.

Modal shoot length at the end of a four week incubation period on medium containing BA : change with season.

Figures 196 to 201.

Explant growth in a four week incubation period on medium containing BA : change with season.


Prunus cerasifera
shoot number
10
8
6
4
2
0
0·1 mgl⁻¹ BA
0·5 mgl⁻¹ BA
1·0 mgl⁻¹ BA
March
April
May
June
July
August
September
October
Month

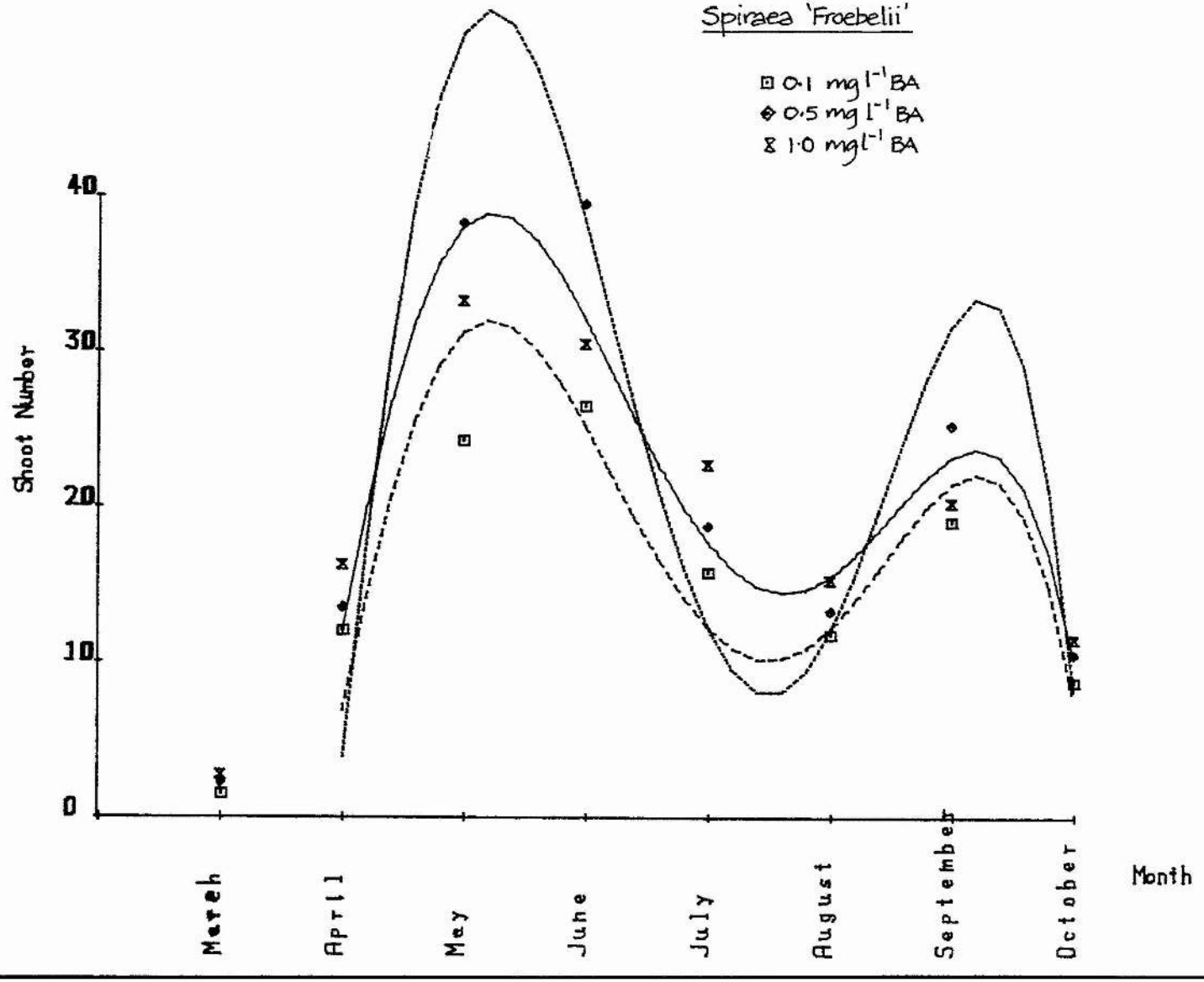

Spiraea 'Froebelii'
0·1 mg l⁻¹ BA
0·5 mg l⁻¹ BA
1·0 mg l⁻¹ BA
Shoot Number
0
10
20
30
40
March
April
May
June
July
August
September
October
Month

Fig 190. Prunus 0.1 mg l^{-1}BA.

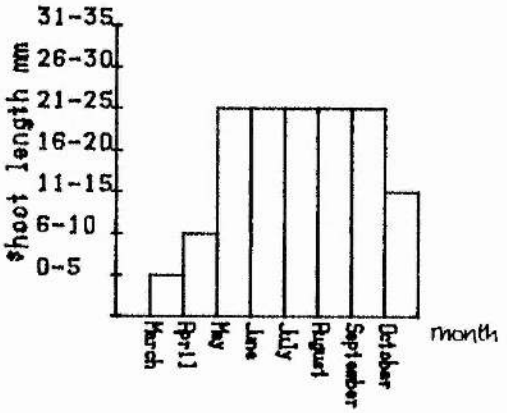


Fig 191. Prunus 0.5 mg l^{-1}BA.

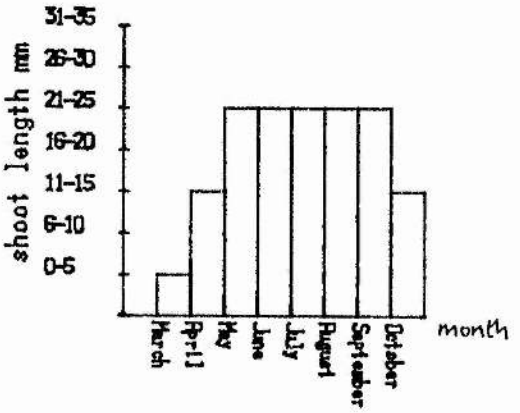


Fig 192. Prunus 1.0 mg l^{-1}BA.

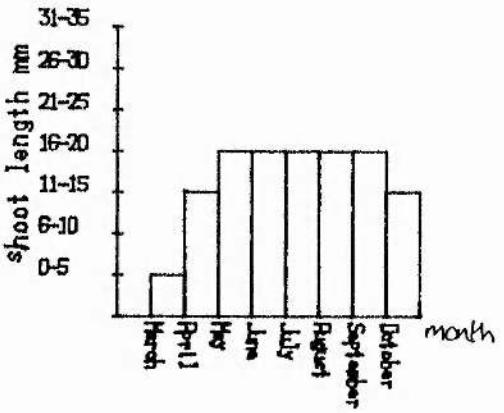


Fig 193. Spiraea 0.1 mg l^{-1} BA.

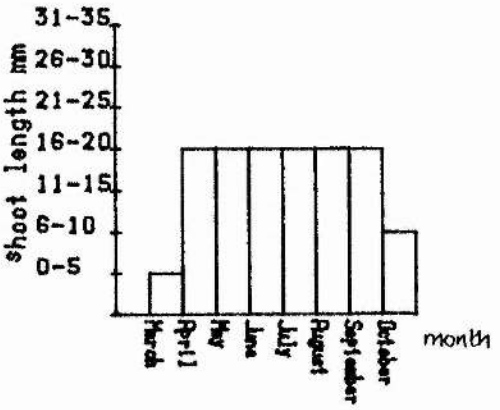


Fig 194. Spiraea 0.5 mg l^{-1} BA.

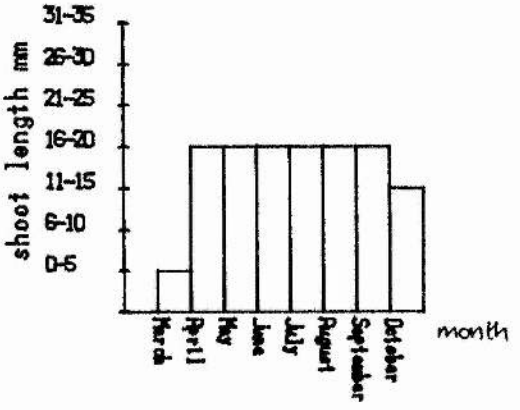


Fig 195. Spiraea 1.0 mg l^{-1} BA.

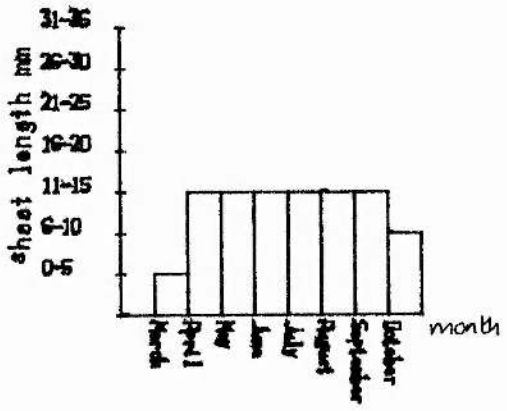


Fig 196. Prunus 0.1 mg l^{-1} BA.

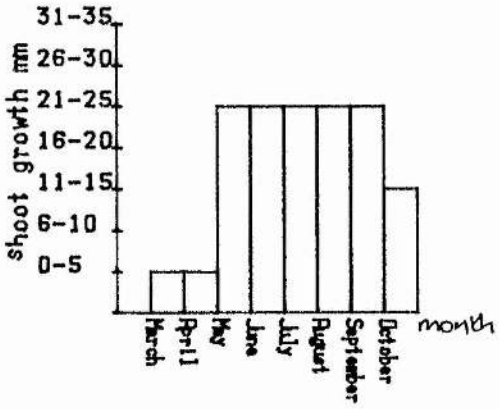


Fig 197. Prunus 0.5 mg l^{-1} BA.

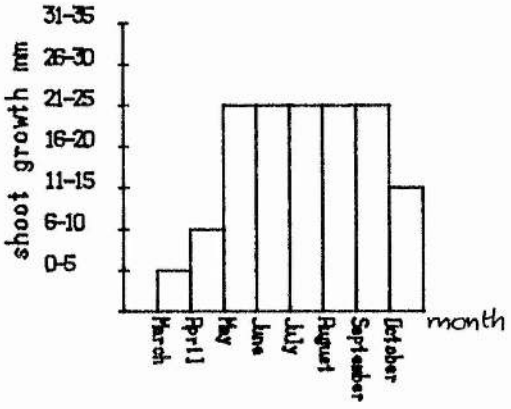


Fig 198. Prunus 1.0 mg l^{-1} BA.

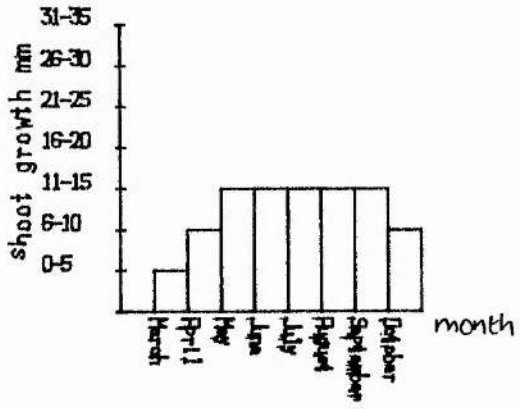


Fig 199. Spiraea 0.1 mg l^{-1} BA.

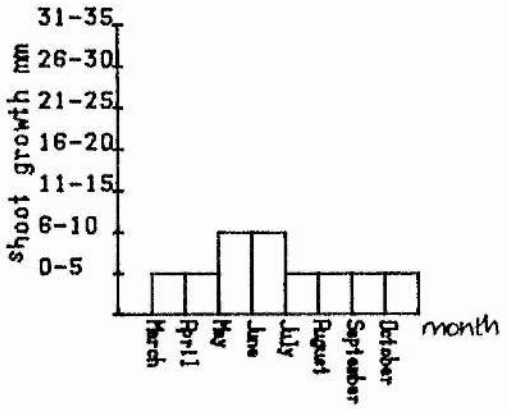


Fig 200. Spiraea 0.5 mg l^{-1} BA.

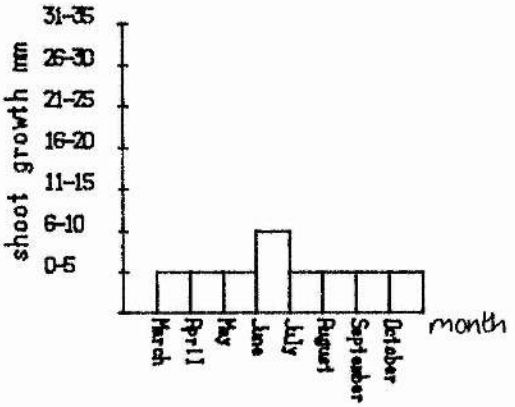


Fig 201. Spiraea 1.0 mg l^{-1} BA.

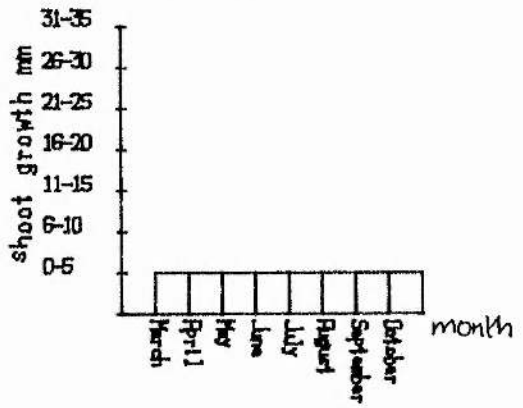


Table 53.

Mean shoot number after a 4 week culture period on medium containing BA : month of culture.

MONTH	SHOOT NUMBER
March	1.29
April	8.42
May	18.21
June	18.38
July	10.67
August	7.33
September	11.96
October	5.58

L.S.D. = 0.76 ($P<.05$)

Table 54.

Analysis of variance for data presented in Figures 188 and 189.

SOURCE	S.S.	D.F.	M.S.	F	P
Month	5912.083	7	844.583	496.408	<.001
Concentration of BA	320.448	2	160.224	94.172	<.001
Species	11750.028	1	11750.028	6906.135	<.001
Month * concentration	373.135	14	26.653	15.665	<.001
Month * species	3365.479	7	480.783	282.583	<.001
Concentration * species	197.572	2	98.786	58.062	<.001
Month * concentration * species	224.177	14	16.013	141.291	<.001
Error	245.000	144	1.701		
Total	22387.917	191			

Table 55.

Mean shoot number after a 4 week culture or subculture period in May and August.

CONCEN-TRATION BA mg l^{-1}	1st CULTURE MAY	SUB-CULTURE JUNE	1st CULTURE AUGUST	SUB-CULTURE SEPTEMBER	MEAN
0.5	38.5	44.0	13.25	30.50	31.50
1.0	33.25	66.00	15.25	46.50	40.25
2.5	34.00	47.50	11.50	27.25	30.06
MEAN	35.17	52.50	13.33	34.75	33.94

L.S.D. = 5.93 (body of table)

L.S.D. = 3.42 (month means)

L.S.D. = 2.96 (variety means)

($p<.05$)

Table 56.

Mean increase in shoot number on subculture of May and August explants.

May culture : June culture

36.0

August culture : September culture

85.7

The difference is significant ($p<.05$).

3.23 DISCUSSION

Explant size

Number of shoots increased with length of the explant. Murashige (1977c) also noted that shoot multiplication rates are usually dependent on initial explant size. This may reflect an increase in the number of sites for shoot initiation with increase in length. However, short explants initiated more shoots per mm than long explants, thus showing that sites for shoot initiation may be fewer as distance from the apex increases. A supporting observation was also noted - in long explants, shoots were more predominant at the apical end of the explant than at the basal end.

Number of sites for shoot initiation may be limited by number of suitable cells, by growth regulators, nutrient or carbohydrate availability. Nutrients and carbohydrate were supplied in the medium and therefore are unlikely to be limiting factors. However, nutrient, carbohydrate and cytokinin uptake could limit shoot formation. Large explants have a greater surface area in contact with the medium than short explants and therefore might be expected to have greater absorption. However, Heller (1965) showed that

less absorption takes place in explants laid horizontally than in vertically oriented explants, and so surface contact area may not account for the observed differences. The length of the explant could however affect capillary uptake.

Shoot length was reduced in very short explants and initial explant growth was also less in short explants. Murashige (1977c) also reported that shoot growth is greater in large than in small explants. This indicates that a factor essential for growth is also lacking in small explants.

As high concentrations of auxin are present at the shoot apex, and auxin is involved in both differentiation and growth, it is possible that auxin is the factor limiting the response in short explants. Since the greatest auxin concentration in the explant is likely to be at the apex, auxin distribution could account for the observed differences in differentiation and shoot length. Although short explants formed fewer shoots than long explants, short shoots formed more shoots per millimetre than long shoots and this observation lends support to the hypothesis that endogenous auxin content determines shoot initiation.

As no differences in shoot initiation or growth

between different cytokinin treatments were observed, cytokinin is unlikely to limit differentiation in short explants.

Presence of axillary buds

Shoot number and length decreased as bud number increased. This shows that axillary buds contain a factor which is inhibitory to both shoot formation and elongation. Auxin is present in buds at high concentrations and therefore could be the inhibitory substance. Other workers have shown auxin to be inhibitory to shoot formation and growth at high concentrations.

Presence of shoot apex

More shoots were formed at low BA concentrations when the apex had been excised. This suggests that an inhibitor may be present in the apex. If this substance is auxin, then these results support the hypothesis that high auxin concentrations inhibit shoot differentiation in the species examined. However, at higher BA concentrations, there was no significant difference between shoot number in the presence and absence of the apex. This shows that, if an inhibitor

is present in the apex, then the effect of the inhibitor can be overcome by cytokinin application. These results therefore support the hypothesis that auxin is the inhibitor responsible for decrease in shoot initiation and thus demonstrate that auxin / cytokinin ratio is important in controlling differentiation.

Extension growth of the initial explant was inhibited by removal of the apex. This is probably due to removal of the apical meristem. However, new shoots were longer if the apex had been excised. This may be due to removal of apical dominance exerted by the apex.

Derivation of explant

Explants from the tops of plants formed a greater number of shoots than those from the base. This suggests that a gradient in a shoot promoter from top to base of the plant or in an inhibitor from base to top is important in determining shoot formation. In the above discussion, auxin was proposed to be an endogenous inhibitor of shoot initiation. These results support this, as auxin is polarly transported from top to base of the plant resulting in greater auxin concentrations at the base of the stem.

An alternative possibility is that basal parts of the plant receive less light than the top of the plant. Lack of light can lead to etiolation of shoots, and etiolated shoots have been shown to have a higher capacity for root formation than non-etiolated shoots (Ryan, 1969). However, shoots used in the experiments discussed here were not etiolated.

Shoot length was greater in explants from the top of the parent plant than in those from the base. This shows that the factor limiting differentiation also inhibits growth. At high BA concentrations, the inhibitory effect on shoot length was eliminated. This data therefore is in support of auxin being the inhibitory substance. The same concentration of inhibitor inhibits both shoot initiation and shoot growth in the test species. However, auxin usually affects differentiation and growth at different concentrations (Thimann, 1977). This could indicate that the inhibitor is not auxin.

Seasonal variation

There was considerable seasonal variation in shoot formation pattern. Shoot differentiation was greatest in spring and early summer but declined markedly in mid

summer and in autumn. A small rise in shoot formation was evident in September.

Other workers have examined the pattern of root formation with season. This is very similar to the pattern for shoot initiation (see Chapter 4). Therefore, similar endogenous factors may control both shoot and root formation. These factors may be hormonal as auxin, cytokinin, gibberellin and abscisic acid have been shown to fluctuate with season (see references in Chapter 4). Inhibitors and phenolic compounds (Hess, 1961) are also likely to be important.

It should be noted that shoot growth did not parallel the recorded increase in shoot initiation in September. This shows that the factor responsible for this increase in differentiation is unlikely to be involved in promotion of growth.

When shoots were subcultured, shoot initiation in August-cultured explants increased more than in May-cultured explants. This shows that the conditions of culture can, after one culture passage, reduce the inhibitory seasonal effect. This could indicate that an inhibitor present in August-cultured explants is reduced or inactivated during the first culture period,

or that the inhibitor is not translocated from the original explant to new shoots (see also Section 3.34).

Summary

From results presented in this section, it can be concluded that endogenous factors, other than cytokinin, are very important in determining shoot initiation. Without further analytical experimentation, the factors involved in the test species cannot be identified, but some factors likely to be important are auxin, gibberellin, abscisic acid and phenolic compounds. The possible involvement of these factors in differentiation is discussed further in Chapter 4.

3.3 SHOOT MORPHOGENESIS AFTER REPEATED SUBCULTURE

Callus and cell suspension cultures have a high capacity for organogenesis when newly formed from differentiated plant material, but reduction of morphogenic responsivity is evident after repeated subculture (Narayanaswamy, 1977). Shoot cultures have not previously been studied in detail during long-term culture, although Jones and Murashige (1974) reported (without any data to support their statement) that a number of (genetically) deviant plants resulted from repeated subculture of shoots of Aechmea fasciata. Genetic aberrants probably appear spontaneously, but the possibility of more subtle change with propagative generation has not previously been examined. Using the shoot culture system as a model, a series of propagative generations can be used to observe subtle changes in shoot growth and morphogenetic patterns.

If changes in morphogenic potential do occur in long term shoot cultures in vitro, then, an investigation of the factors controlling this change may elucidate control mechanisms of differentiation.

By maintaining similar environmental, nutritional and growth regulator conditions for each generation, the effect of season (found to be important in determining caulogenic potential in Section 3.2) can be eliminated as a causal factor in change. This section investigates the likelihood of change and its causes.

3.31 THE EFFECT OF REPEATED CYTOKININ TREATMENTS

Method

Experiment 1.

The following species were used in the experiment :- *Chaenomeles japonica*, *Crataegus brachyacantha*, *Potentilla* 'Coronation Triumph', *Potentilla* 'Sutter's Gold', *Prunus cerasifera*, *Prunus tomentosa* and *Spiraea* 'Froebelii'.

BA was incorporated in the nutrient medium at 0, 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0 mg l^{-1}.

Shoots were subcultured every four weeks. Shoots were selected from the treatment which had formed the greatest number of shoots during the previous culture period (one shoot per culture tube).

Cultures were incubated in light (16 hour photoperiod). Shoot number, shoot length and initial explant length were recorded at the end of each four week culture period for a total of 9 generations. Presence of callus was also recorded.

Experiment 2.

Nine generations of shoots of Spiraea 'Froebelii' were cultured on medium containing 2iP. 2iP concentrations and culture conditions were the same as for Experiment (2) above. Shoot number and length were recorded at the end of each four week culture period. Presence of callus was also noted.

Results

Experiment 1.

Shoot number increased over the first few generations and then gradually declined in all species at all BA concentrations except in Chaenomeles in which a decline in shoot formation only occurred at 5.0 mg l^{-1} BA. Figures 202 to 2507 show fitted polynomial curves of shoot number plotted against propagative generation. The significance of fit is given beside each figure ($p<.05$ - $p<.001$).

Shoot length decreased with increasing number of subcultures and with increase in BA concentration (Figures 251 to 257). Table 57 shows the significance of the differences in shoot length with propagative generation. An analysis of variance (Table 58) demonstrated a significant effect due to generation $p<.001$), BA concentration ($p<.001$) and species ($p<.001$). There was also a significant interaction between species and generation ($p<.05$).

An increasing incidence of callus was noted in later generations (Table 59). Both the frequency of green leafy callus and yellow/white callus increased after Generation 5 at high BA concentrations in all species except Potentilla (both cultivars) in which no callus formed in any treatment.

Leaf size decreased progressively with repeated subculture (Plates 7 to 12) and darkened in colour to dark green in later subcultures. Leaves of Prunus cerasifera lost their red colour in later generations. Leaves in later subcultures sometimes had a distorted shape, and increasingly in Prunus species particularly, tended to abscind readily. Stems became increasingly brittle with subculture.

Experiment 2.

An initial rise in shoot formation followed by a slight decline was recorded in Spiraea when subcultured repeatedly on medium containing 2iP (Figures 258 to 262). An analysis of variance showed a significant effect due to generation ($p<.001$), a significant effect due to BA concentration ($p<.001$) and a significant interaction between these ($p<.001$) (Table 60). Table 61 shows change in shoot number with propagative generation (combined data for all 2iP concentrations).

A comparison of change in shoot number was made between BA and 2iP cultures. Decrease in shoot number with propagative generation was significantly greater ($p<.001$) in BA treatments than in 2iP treatments (Figure 263).

A slight decrease in shoot length was recorded with subculture on 2iP medium (Figure 264). This decrease was less than was recorded in BA treatments (compare Figure 251).

Figures 202 to 250.

Mean shoot number at the end of each four week subculture period on medium containing BA.

Chaenomeles

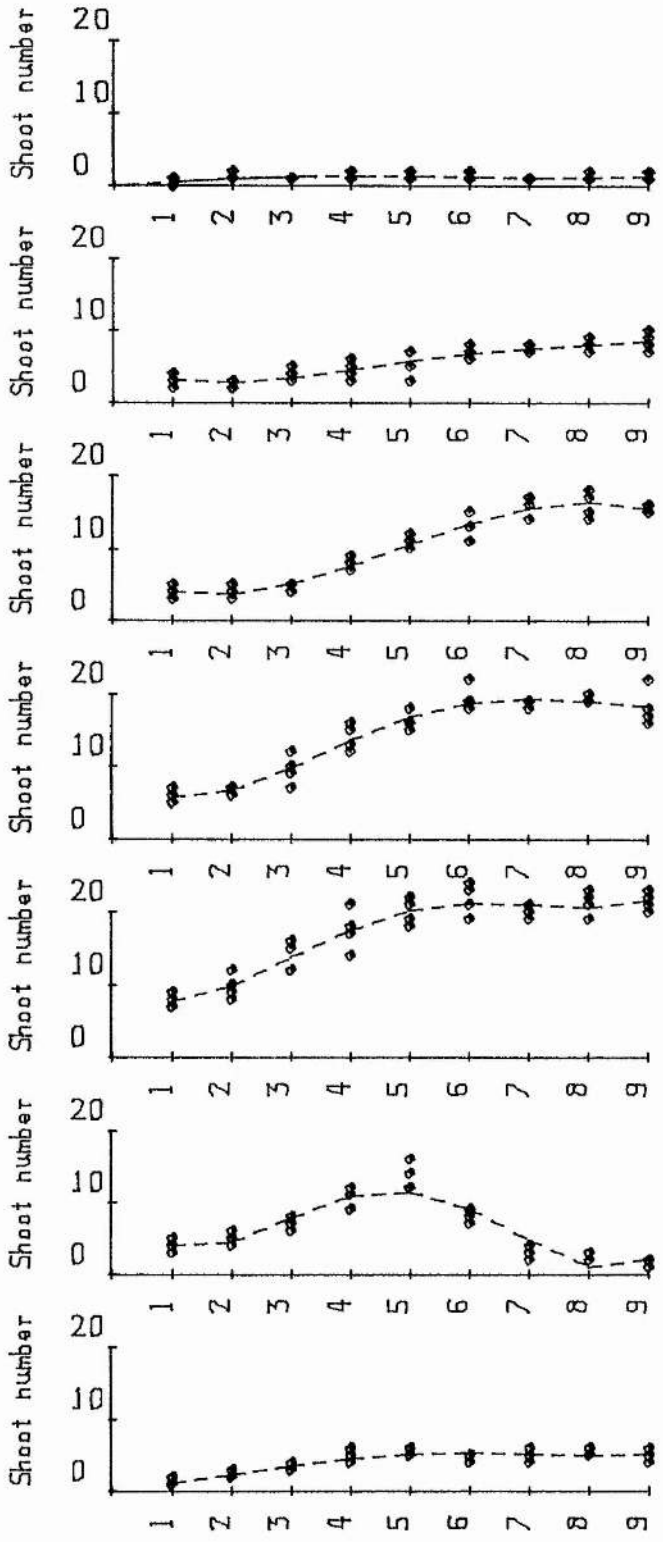


Fig 202. BA 0 mg l^{-1}
r= .4959 ($p<.01$)
Propagative Generation

Fig 203 BA 0.1 mg l^{-1}
r= .9038 ($p<.001$)
Propagative Generation

Fig 204 BA 0.5 mg l^{-1}
r= .9763 ($p<.001$)
Propagative Generation

Fig 205 BA 1.0 mg l^{-1}
r= .9662 ($p<.001$)
Propagative Generation

Fig 206 BA 2.5 mg l^{-1}
r= .9503 ($p<.001$)
Propagative Generation

Fig 207 BA 5.0 mg l^{-1}
r= .9206 ($p<.001$)
Propagative Generation

Fig 208 BA 10.0 mg l^{-1}
r= .9080 ($p<.001$)
Propagative Generation

Crataegus brachyacantha.

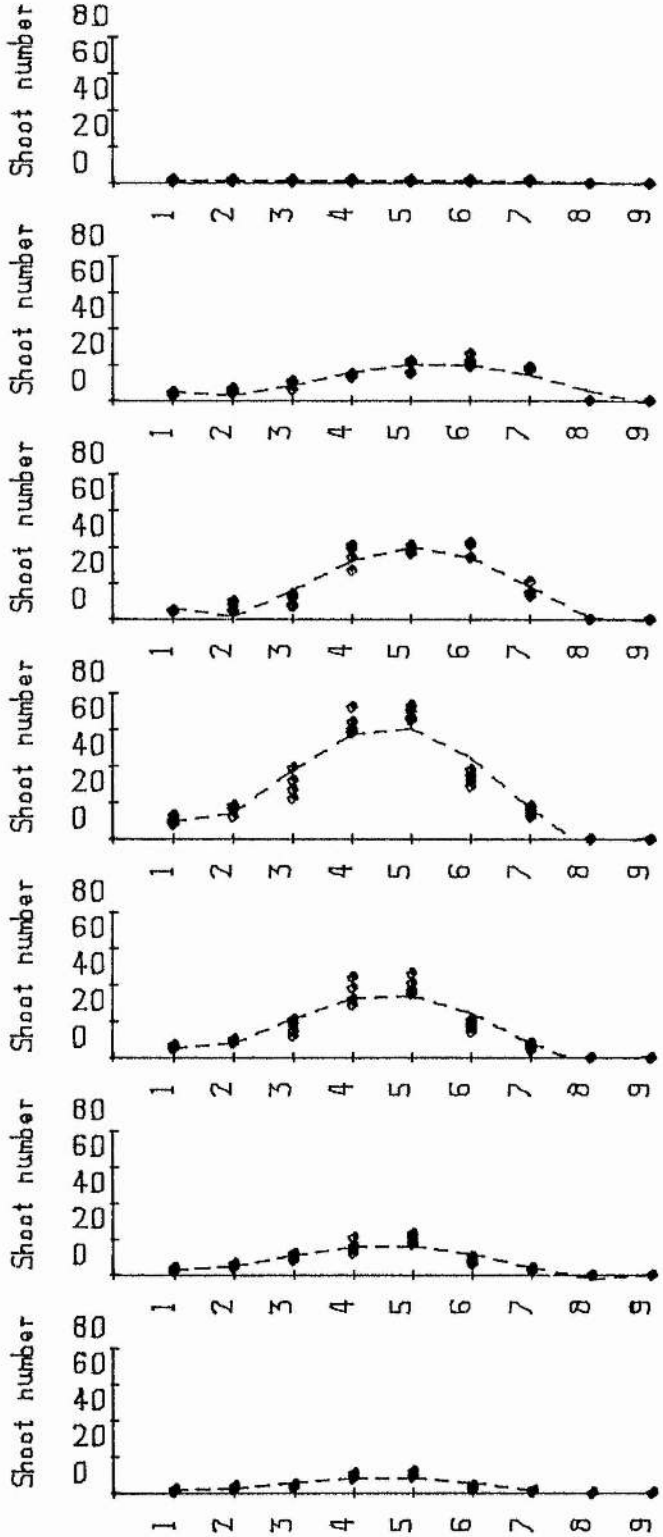


Fig 209 BA 0 mg l^{-1}
r= .7426 ($p<.001$)
Propagative Generation

Fig 210 BA 0.1 mg l^{-1}
r= .9163 ($p<.001$)
Propagative Generation

Fig 211 BA 0.5 mg l^{-1}
r= .9608 ($p<.001$)
Propagative Generation

Fig 212 BA 1.0 mg l^{-1}
r= .9570 ($p<.001$)
Propagative Generation

Fig 213 BA 2.5 mg l^{-1}
r= .9366 ($p<.001$)
Propagative Generation

Fig 214 BA 5.0 mg l^{-1}
r= .9244 ($p<.001$)
Propagative Generation

Fig 215 BA 10.0 mg l^{-1}
r= .8994 ($p<.001$)
Propagative Generation

Potentilla 'Coronation Triumph.'

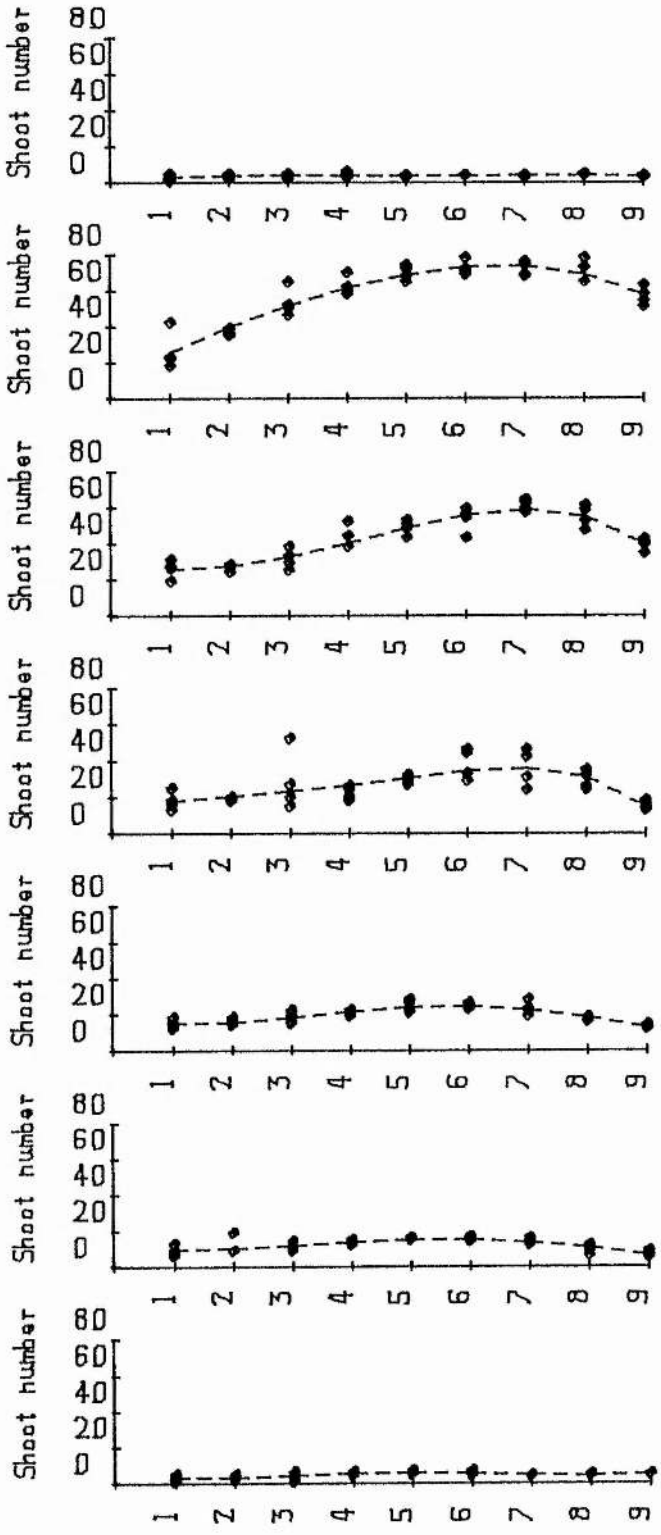


Fig 216 BA 0 mg l^{-1}
r= .3538 ($P<.05$)
Propagative Generation

Fig 217 BA 0.1 mg l^{-1}
r= .9448 ($P<.001$)
Propagative Generation

Fig 218 BA 0.5 mg l^{-1}
r= .9313 ($P<.001$)
Propagative Generation

Fig 219 BA 1.0 mg l^{-1}
r= .7044 ($P<.001$)
Propagative Generation

Fig 220 BA 2.5 mg l^{-1}
r= .8758 ($P<.001$)
Propagative Generation

Fig 221 BA 5.0 mg l^{-1}
r= .7737 ($P<.001$)
Propagative Generation

Fig 222 BA 10.0 mg l^{-1}
r= .6456 ($P<.001$)
Propagative Generation

Potentilla 'Sutter's Gold.

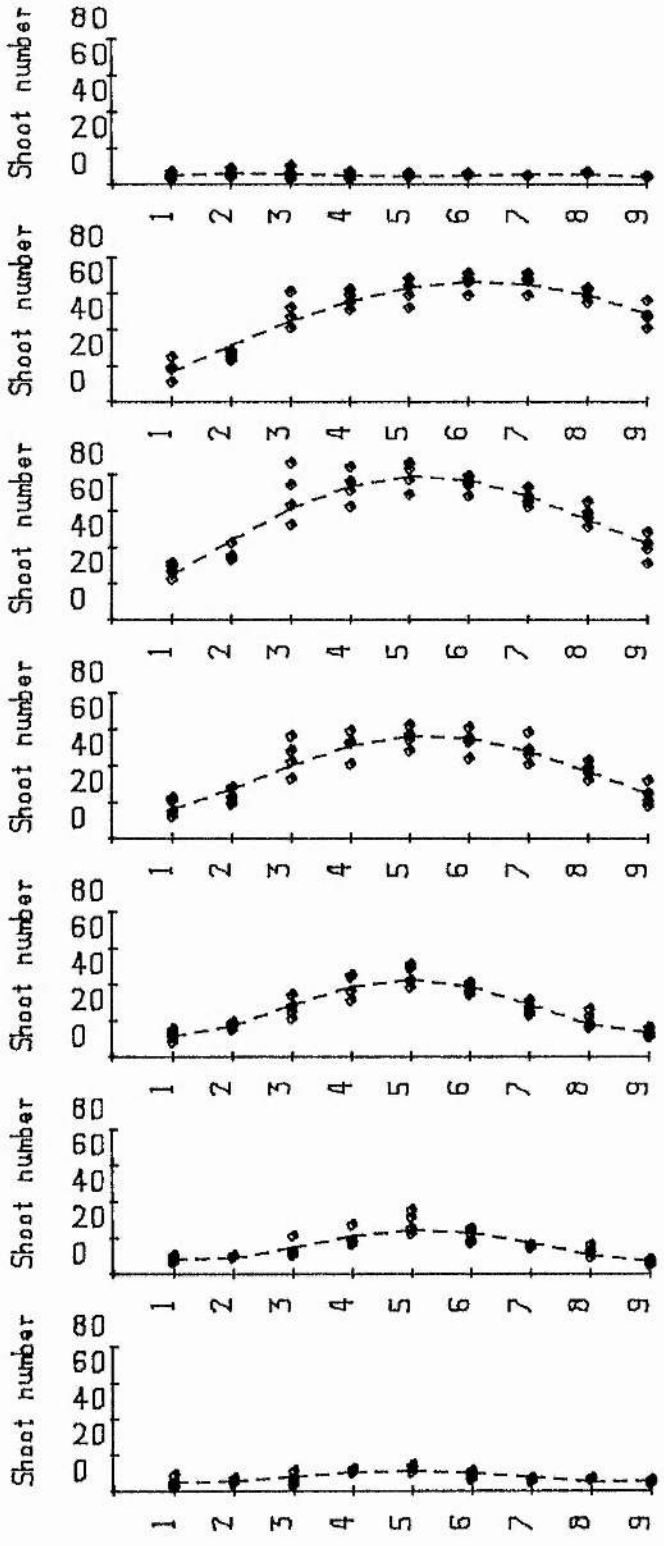


Fig 223. BA 0 mg l^{-1}
r= .3922 ($p<.05$)
Propagative Generation

Fig 224. BA 0.1 mg l^{-1}
r= .9432 ($p<.001$)
Propagative Generation

Fig 225. BA 0.5 mg l^{-1}
r= .9168 ($p<.001$)
Propagative Generation

Fig 226. BA 1.0 mg l^{-1}
r= .9111 ($p<.001$)
Propagative Generation

Fig 227. BA 2.5 mg l^{-1}
r= .9382 ($p<.001$)
Propagative Generation

Fig 228. BA 5.0 mg l^{-1}
r= .8658 ($p<.001$)
Propagative Generation

Fig 229 BA 10.0 mg l^{-1}
r= .7455 ($p<.001$)
Propagative Generation

Prunus ceraslfera.

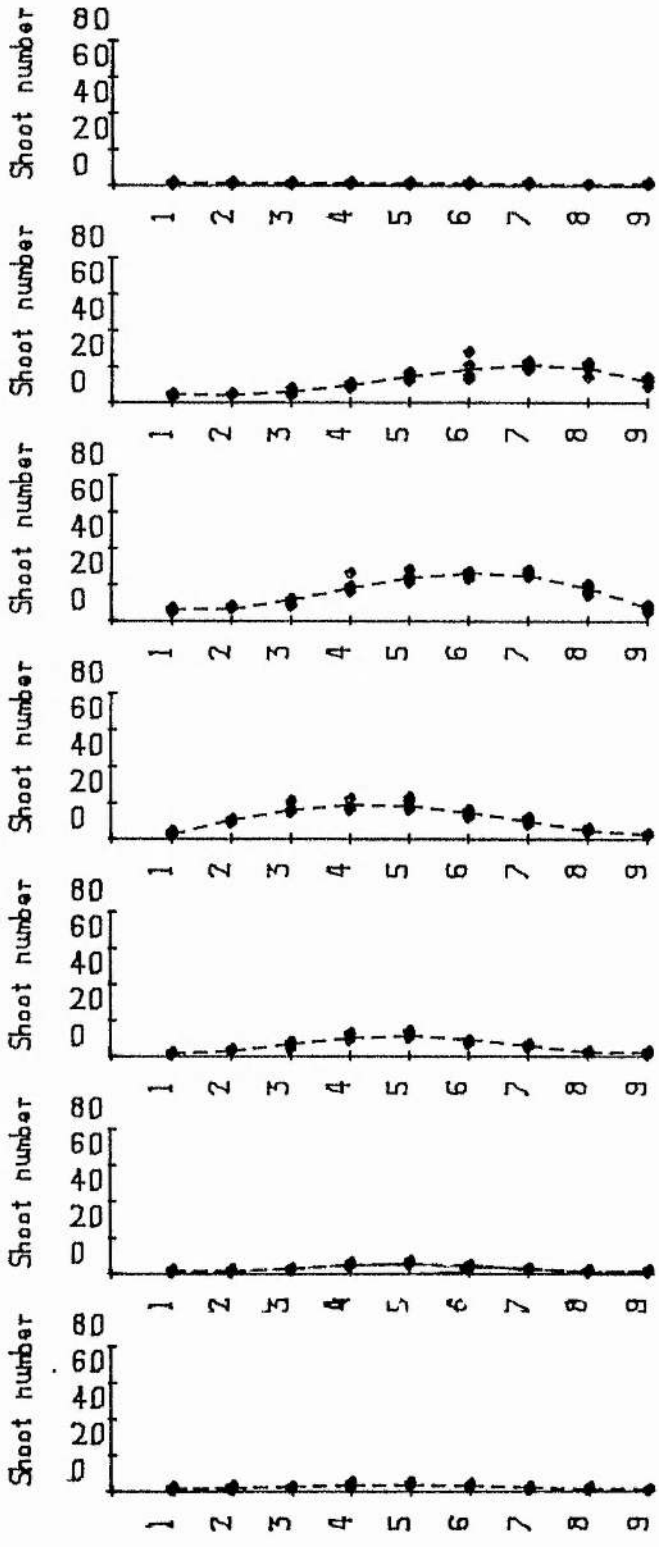


Fig 230. BA 0 mg l^{-1}

r= .3402

Propagative Generation

Fig 231. BA 0.1 mg l^{-1}

r= .9206

Propagative Generation

Fig 232. BA 0.5 mg l^{-1}

r= .9584

Propagative Generation

Fig 233 BA 1.0 mg l^{-1}

r= .9567

Propagative Generation

Fig 234. BA 2.5 mg l^{-1}

r= .9412

Propagative Generation

Fig 235 BA 5.0 mg l^{-1}

r= .9141

Propagative Generation

Fig 236. BA 10.0 mg l^{-1}

r= .76

Propagative Generation

Prunus tomentosa.

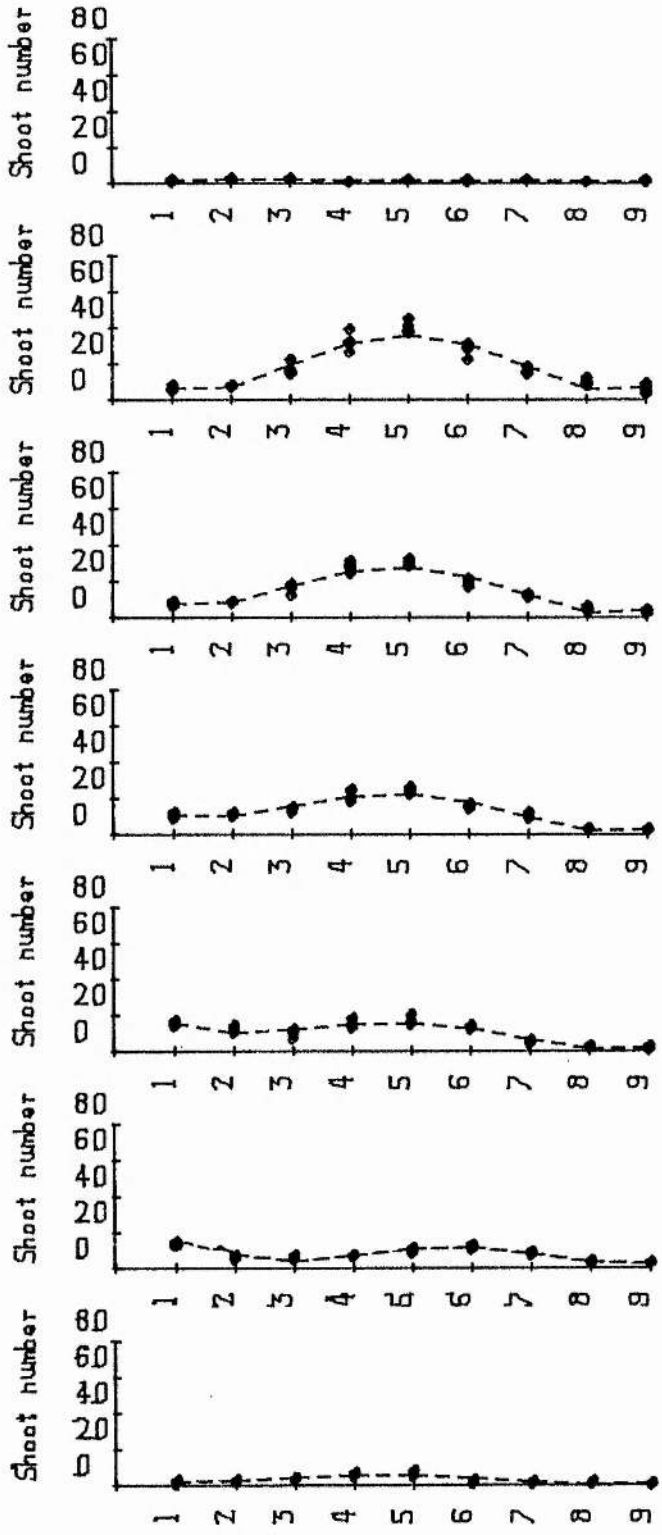


Fig 237. BA 0 mg l^{-1}
r= .5231 ($p<.01$)
Propagative Generation

Fig 238 BA 0.1 mg l^{-1}
r= .9521 ($p<.001$)
Propagative Generation

Fig 239. BA 0.5 mg l^{-1}
r= .9652 ($p<.001$)
Propagative Generation

Fig 240. BA 1.0 mg l^{-1}
r= .9655 ($p<.001$)
Propagative Generation

Fig 241. BA 2.5 mg l^{-1}
r= .9431 ($p<.001$)
Propagative Generation

Fig 242. BA 5.0 mg l^{-1}
r= .9762 ($p<.001$)
Propagative Generation

Fig 243. BA 10.0 mg l^{-1}
r= .8286 ($p<.001$)
Propagative Generation

Spiraea 'Froebelii'.

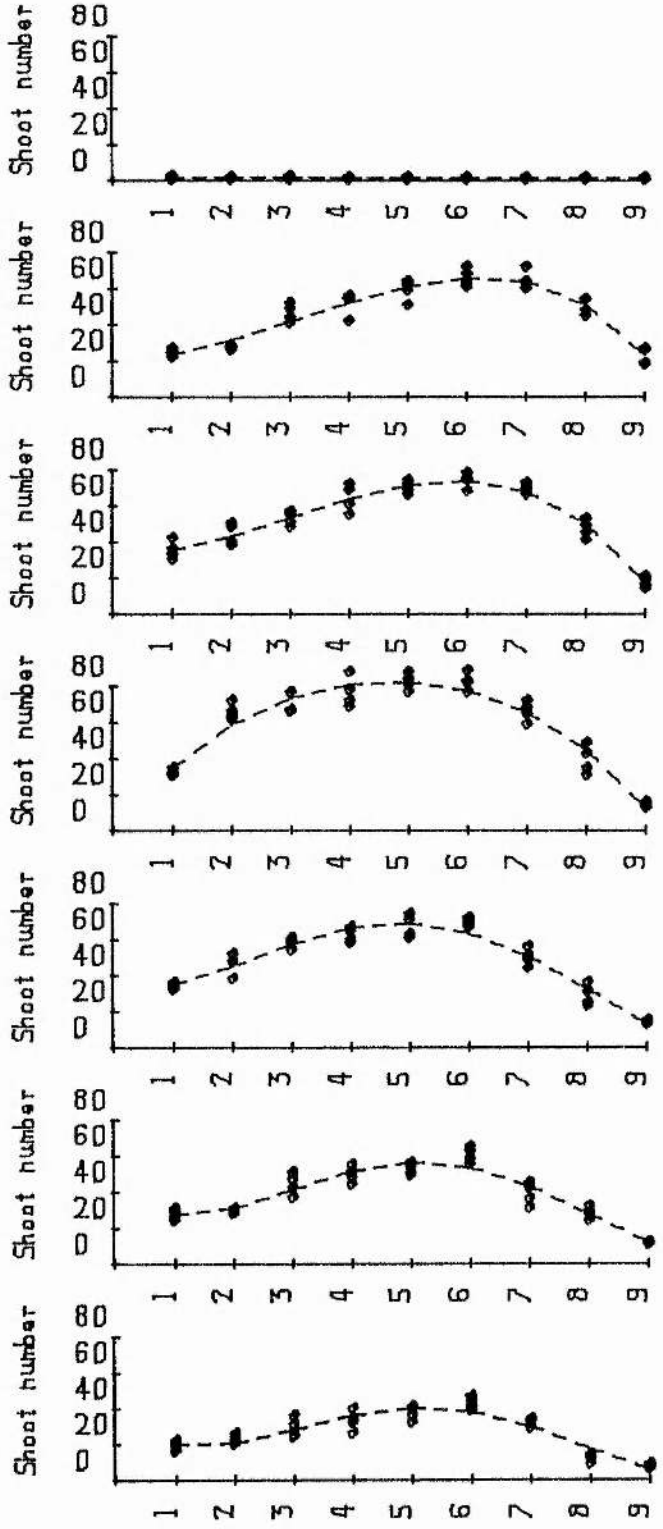


Fig 244. BA 0 mg l^{-1}
r= .2298 N.S.

Propagative Generation

Fig 245. BA 0.1 mg l^{-1}
r= .9593 ($p<.001$)

Propagative Generation

Fig 246. BA 0.5 mg l^{-1}
r= .9703 ($p<.001$)

Propagative Generation

Fig 247. BA 1.0 mg l^{-1}
r= .9639 ($p<.001$)

Propagative Generation

Fig 248. BA 2.5 mg l^{-1}
r= .9651 ($p<.001$)

Propagative Generation

Fig 249. BA 5.0 mg l^{-1}
r= .9458 ($p<.001$)

Propagative Generation

Fig 250. BA 10.0 mg l^{-1}
r= .9240 ($p<.001$)

Propagative Generation

Figures 251 to 257.

Modal shoot length at the end of each four week subculture period on medium containing BA.

Fig 251. Chaenomeles japonica.

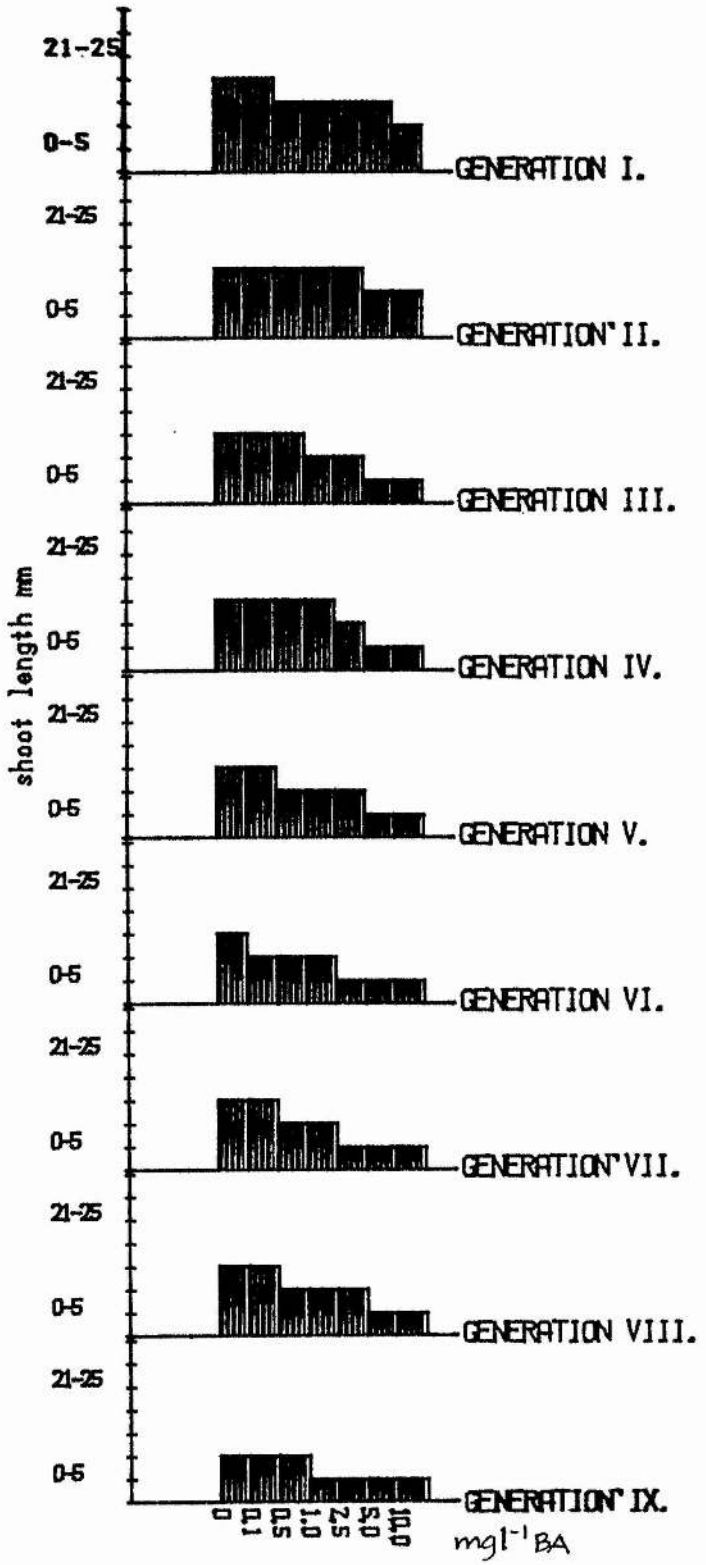


Fig 252. Crataegus bracyacantha.

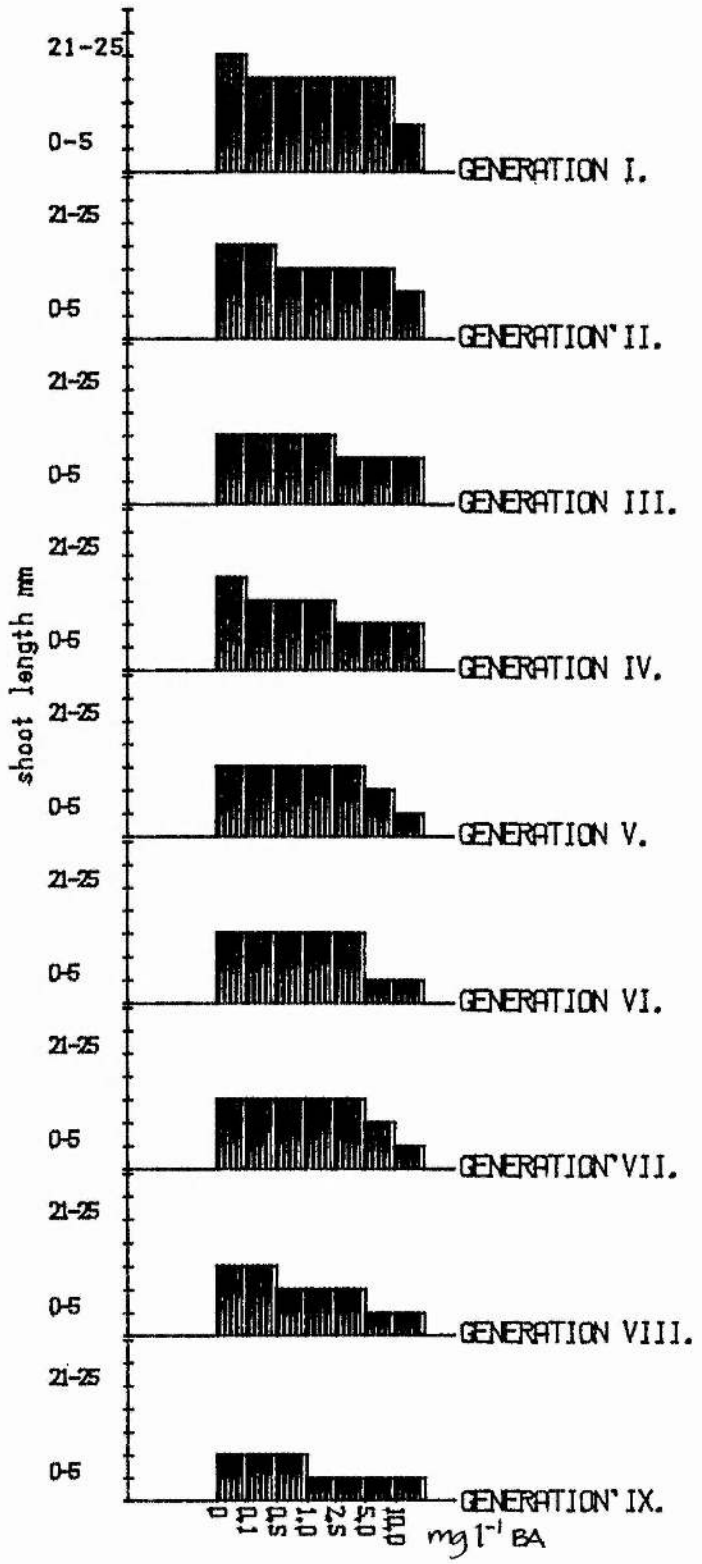


Fig 253 Potentilla 'Coronation Triumph'.

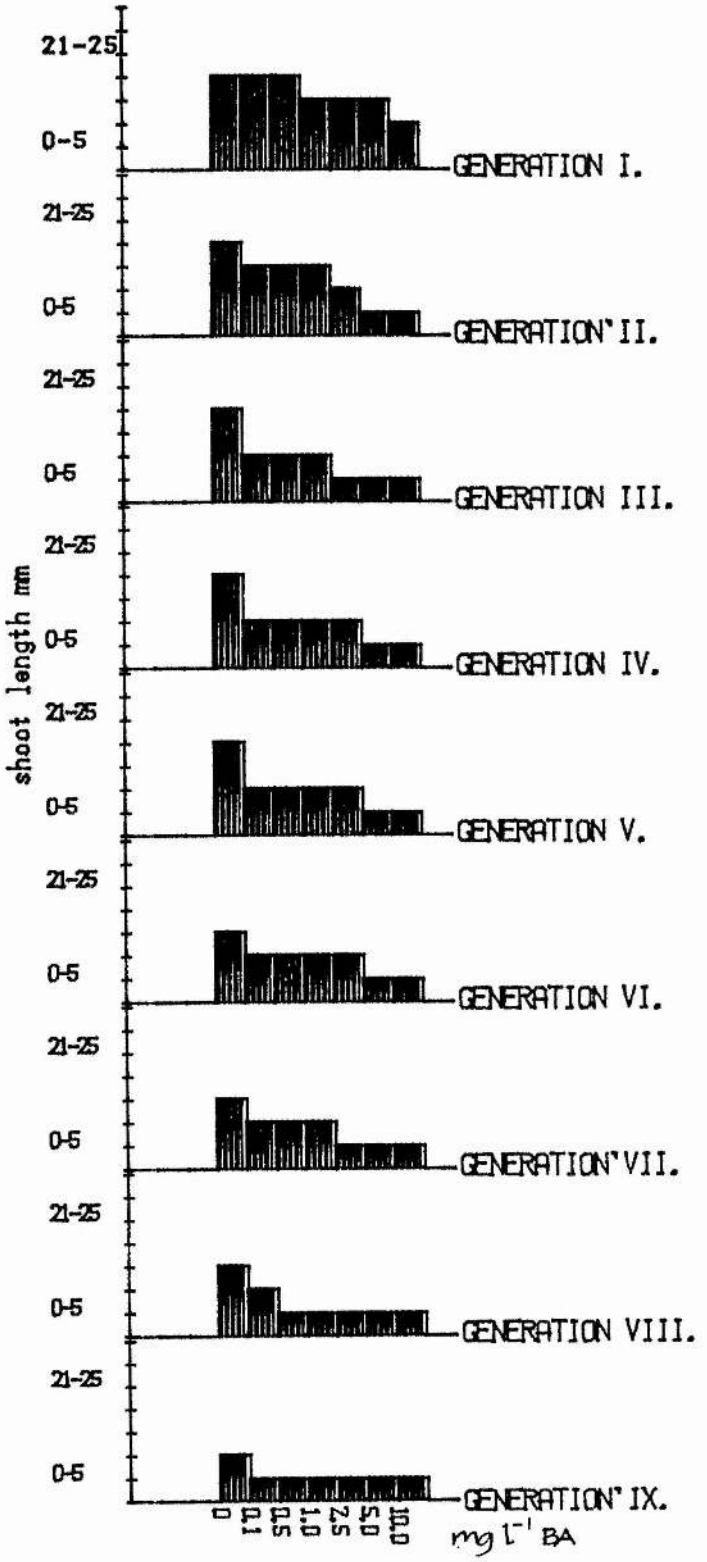


Fig 254 Potentilla 'Sutter's Gold'.

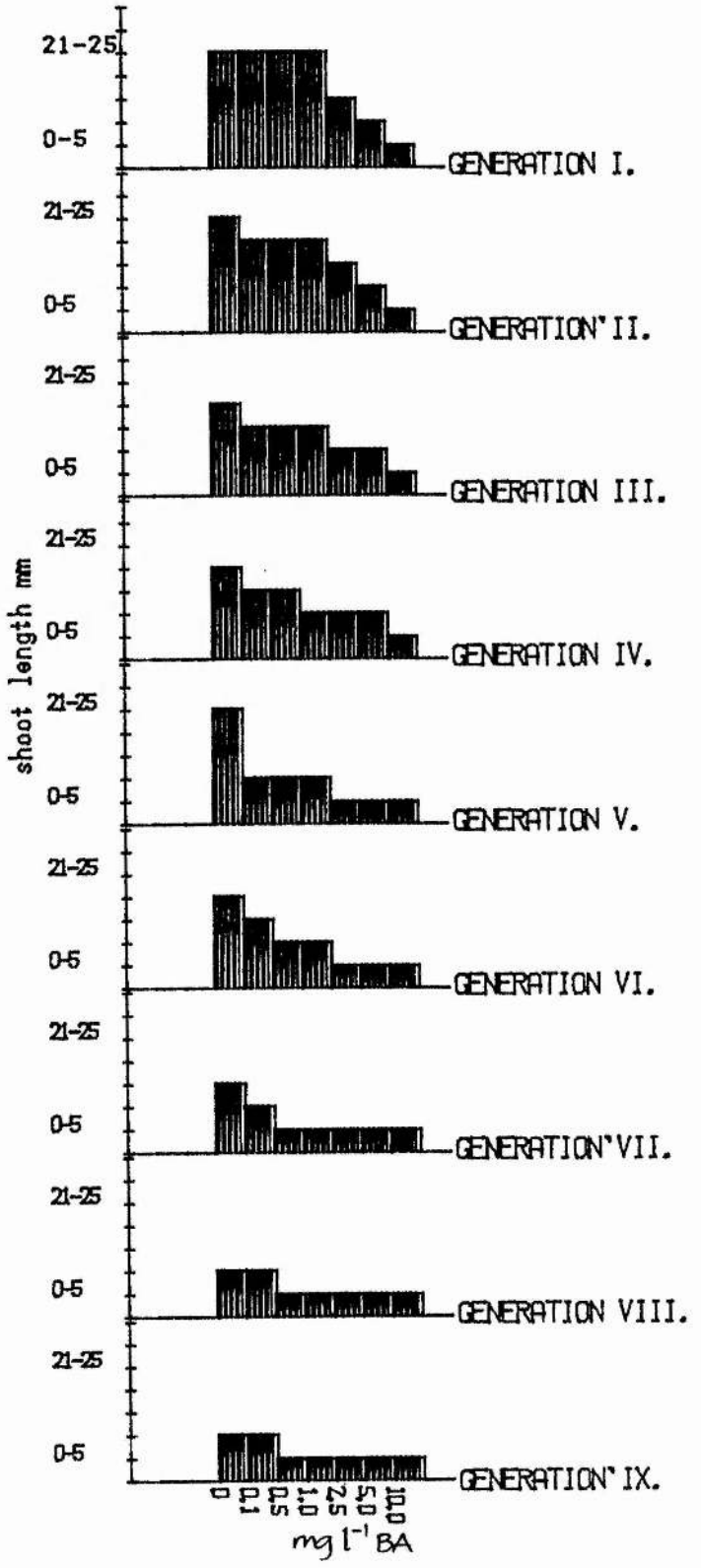


Fig 255 Prunus cerasifera.

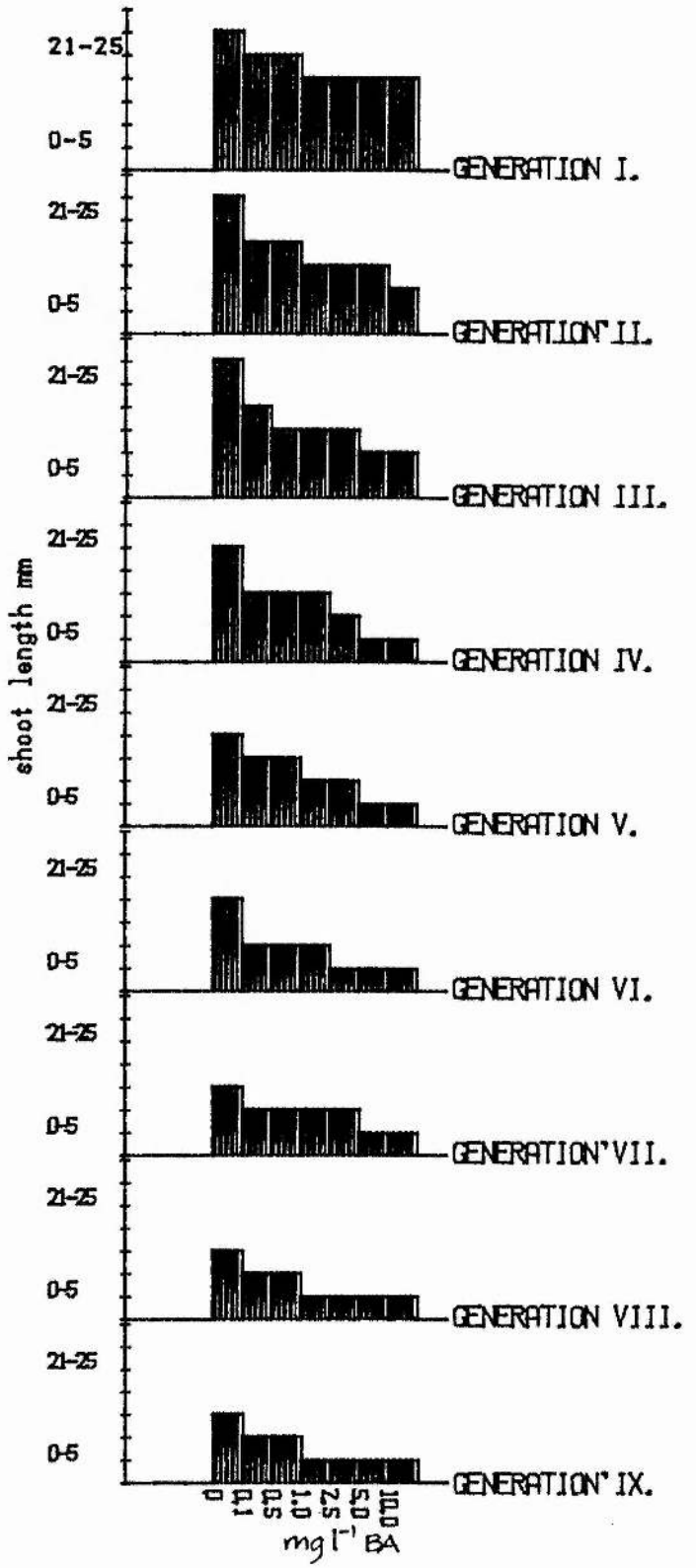


Fig 256. Prunus tomentosa.

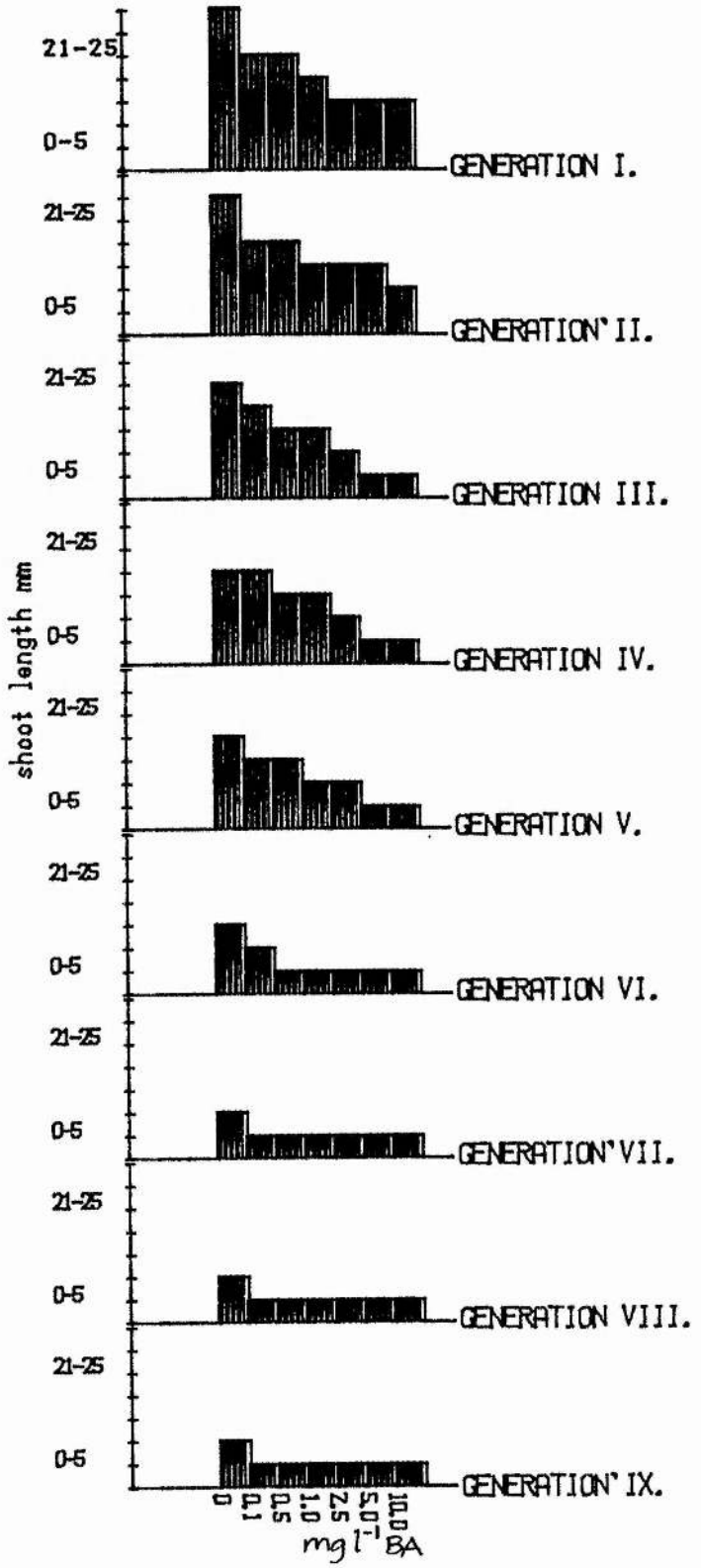


Fig 25. Spiraea 'Froebelii'

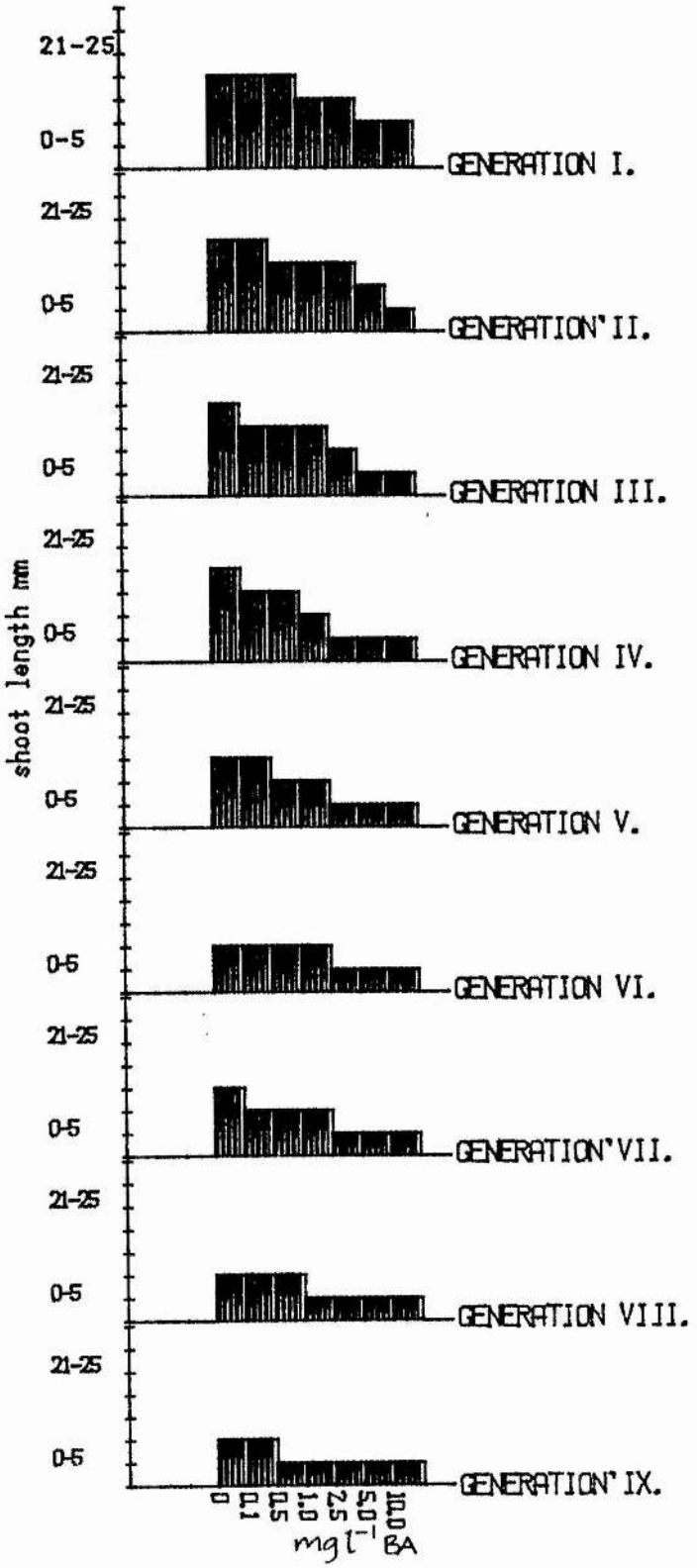


Table 57.

Change in shoot length with propagative generation (combined data for all species and concentrations).

Generation	Shoot length
1	16.72a
2	13.41b
3	10.55c
4	9.94cd
5	8.71d
6	7.18e
7	6.88e
8	4.61f
9	3.89f

($p<.05$)

Table 58.

Analysis of variance for data presented in Figures 251 to 257.

SOURCE	S.S.	D.F.	M.S.	F	P
Species	5785.006	6	964.168	108.522	<.001
Generation	6637.719	8	829.715	93.389	<.001
Interaction	686.678	48	14.306	1.610	<.05
Error	3358.357	378	8.884		
Total	16467.760	440			

Plate 7.

Shoot culture of Crataegus brachyacantha - generation 3.

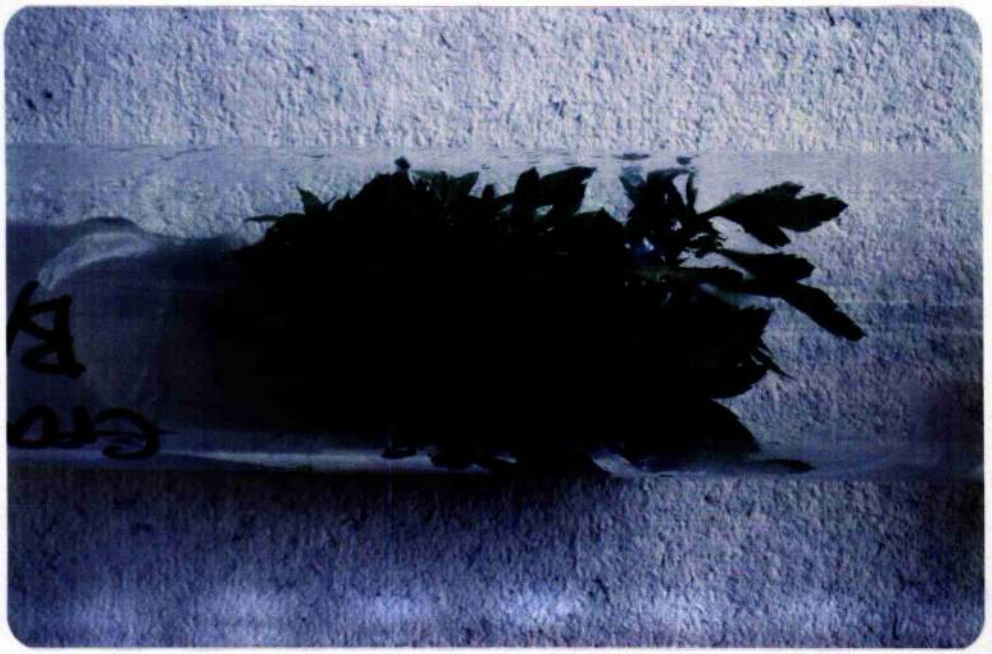


Plate 8.

Shoot culture of Crataegus brachyacantha - generation 7.

Plate 9.

Reduction of leaf size and shoot length with propagative generation - Chaenomeles japonica.

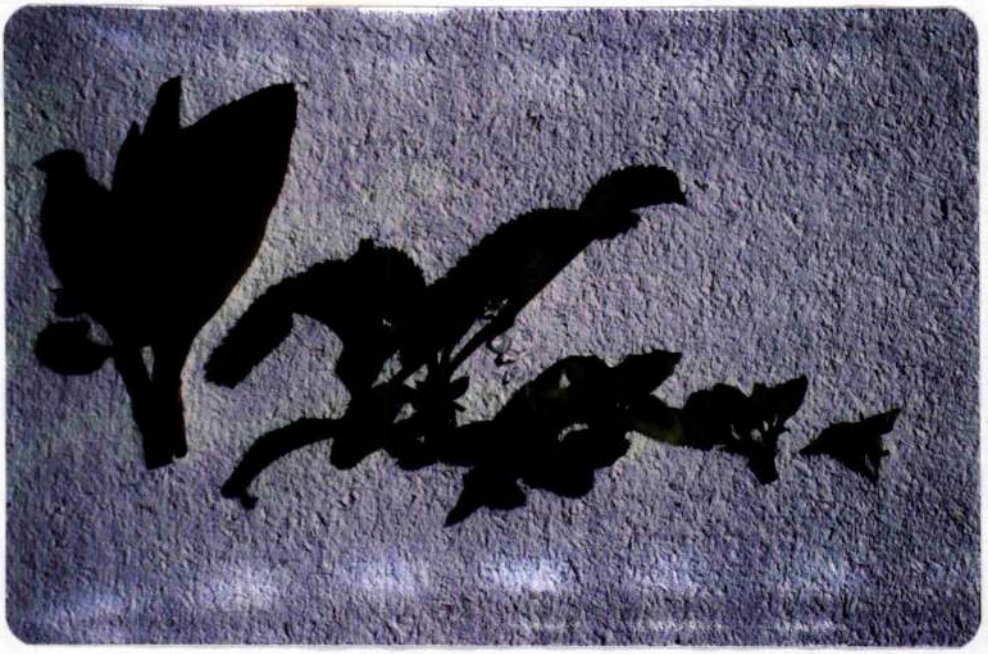


Plate 10.

Reduction of leaf size and shoot length with propagative generation - Cotoneaster dammeri.

Plate 11.

Reduction of leaf size and shoot length with propagative generation - Crataegus brachyacantha

Plate 12.

Reduction of leaf size and shoot length with propagative generation - Potentilla 'Coronation Triumph'

Table 59. Presence of callus in shoot cultures:

Chaenomeles japonica.

Generation	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
1		*	*	*	*		
2				*	*		
3						*	
4						*	*
5					*	*	
6					*	*	
7					*	*	
8					*	*	
9						*	*

Crataegus brachyacantha.

Generation	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
1					*	*	
2						*	
3							*
4							*
5						*	
6						*	
7			*	*	*	*	
8	*	*	*	*	*	*	
9	*	*	*	*	*	*	*

Table 59 continued. Presence of callus in shoot cultures: Prunus cerasifera.

Generation	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
1				*	*	*	
2						*	*
3						*	*
4							*
5						*	
6				*	*	*	
7			*	*	*	*	
8			*	*	*	*	
9			*	*	*	*	*

Prunus tomentosa.

Generation	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
1				*	*		
2							*
3							
4							
5							
6					*	*	
7				*	*	*	
8			*	*	*	*	*
9		*	*	*	*	*	*

Table 59 continued. Presence of callus in shoot cultures: Spiraea 'Froebelii'.

Generation	BA concentration (mg l^{-1})						
	0	0.1	0.5	1.0	2.5	5.0	10.0
1					*	*	
2							*
3							
4							
5							
6							
7						*	
8						*	*
9				*	*	*	*

Figures 258 to 262.
Mean shoot number at the end of each four week subculture period on medium containing 2iP : Spiraea 'Froebelii'.

Figure 263.
Comparison of change in shoot number with propagative generation between BA and 2iP incubation : Spiraea 'Froebelii'.

Linear correlation coefficient
2iP $r = -.7192$ ($p < .001$)
BA $r = -.9793$ ($p < .001$)

Comparison of regression coefficients
2iP $b = -1.700$
BA $b = -23.275$
$p < .001$

Figure 264.
Modal shoot length at the end of each four week subculture period on medium containing 2iP ; Spiraea 'Froebelii'

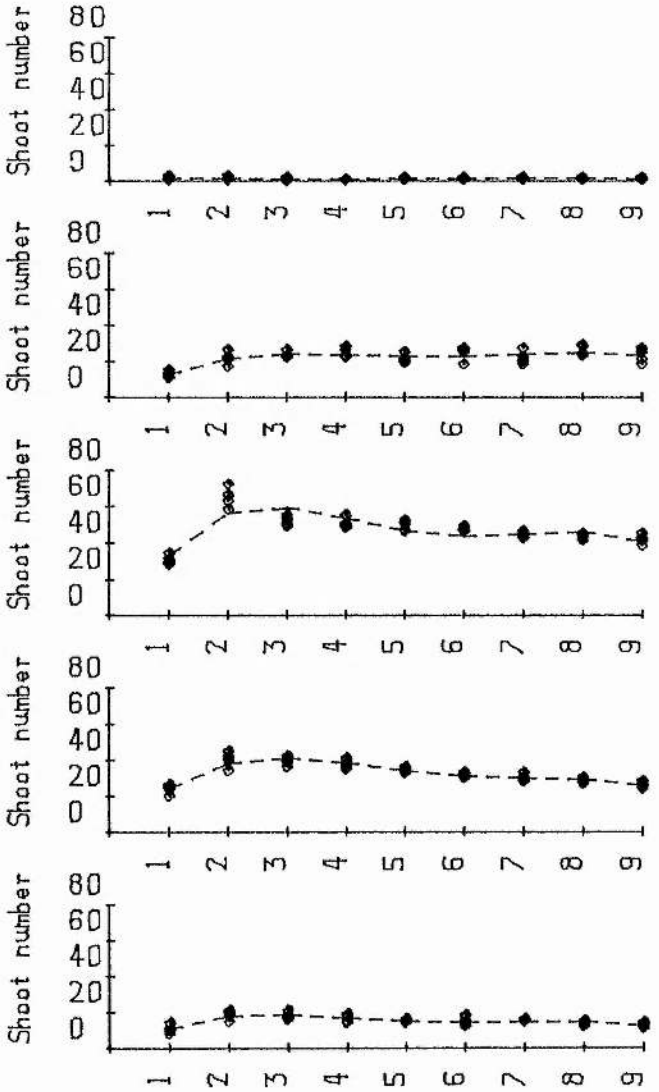


Fig 258. 2iP 0 mg l^{-1}

r= .3222

Propagative Generation

Fig 259. 2iP 5 mg l^{-1}

r= .7587

Propagative Generation

Fig 260. 2iP 10 mg l^{-1}

r= .8349

Propagative Generation

Fig 261. 2iP 15 mg l^{-1}

r= .9196

Propagative Generation

Fig 262. 2iP 20 mg l^{-1}

r= .7886

Propagative Generation

Fig 263. Spiraea 'Froebelii'.

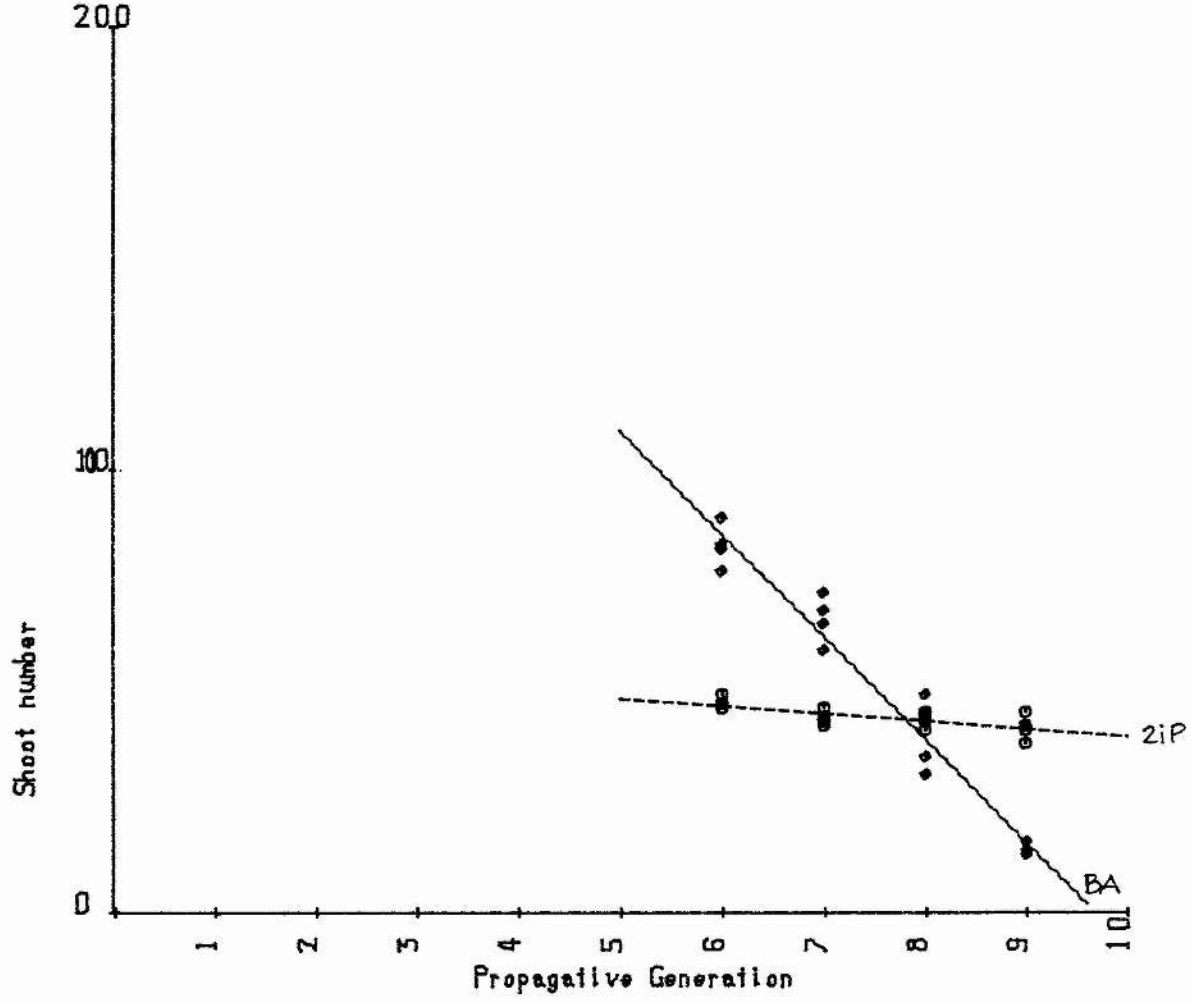


Fig 264 Spiraea 'Froebelii'.

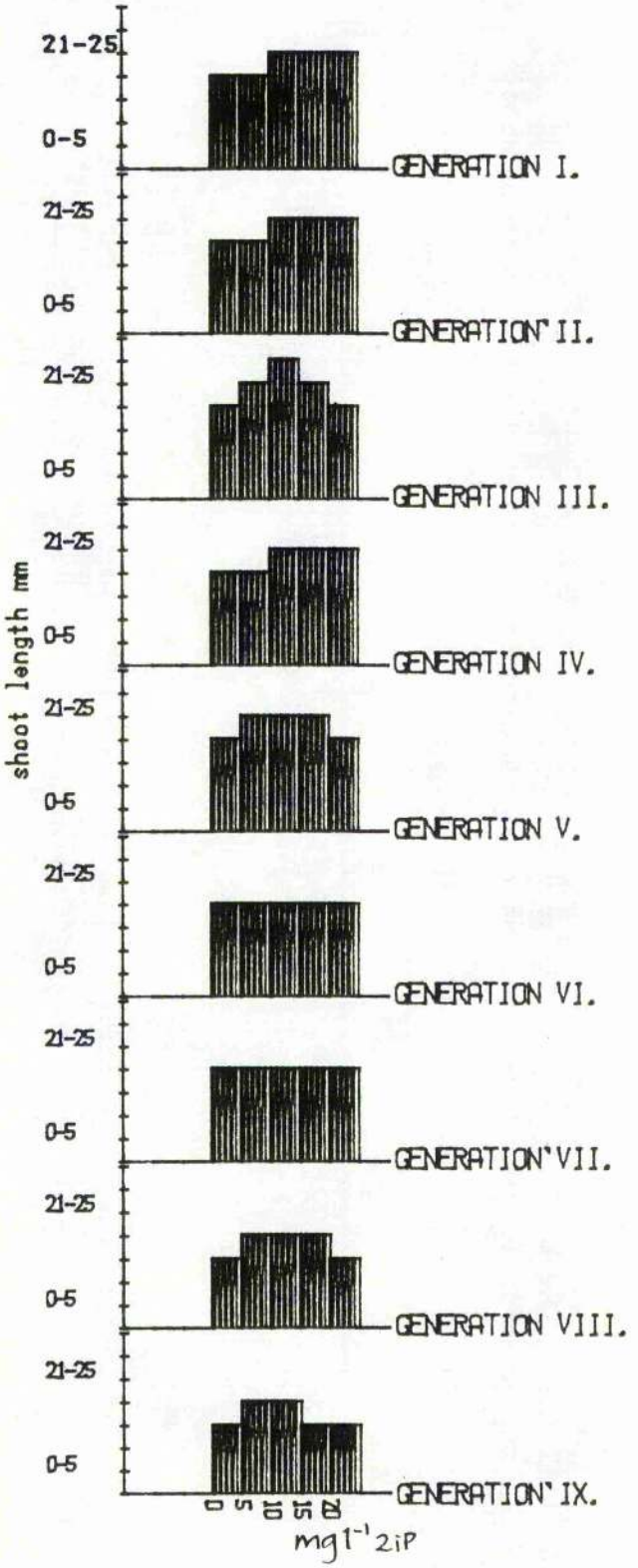


Table 58.

Analysis of variance for data presented in Figures 258 to 262.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP concentrati	42757.633	4	10689.408	1796.539	<.001
Generation	23289.244	8	298.656	50.194	<.001
Interaction	2146.867	32	67.090	11.276	<.001
Error	803.250	135	5.950		
Total	48096.995	179			

Table 61

Change in shoot number with propagative generation when cultured on medium containing 2iP : Spiraea 'Froebelii'.

Generation	Shoot number
1	15.8f
2	29.4a
3	26.7b
4	26.1b
5	24.1c
6	23.7cd
7	22.5cd
8	22.5d
9	20.9e

(p<.05)

3.32 TREATMENT OF REPEATEDLY SUBCULTURED SHOOTS WITH GIBBERELLIN, AUXIN OR CYTOKININ

The previous section (3.31) demonstrated that shoot length and leaf size decreased with increasing number of subcultures on medium containing BA. Auxin and gibberellin promote shoot elongation and leaf shape and size have been shown to be controlled by gibberellin / cytokinin ratio (Mauseth and Halperin, 1975; Engelke et al., 1973). Section 3.1 demonstrated that 2iP could promote shoot elongation. IBA, GA and 2iP were therefore applied to eighth generation shoots to determine (1) whether decrease in shoot and leaf size is reversible and (2) whether the decrease could be due to auxin, gibberellin or endogenous cytokinin de ficiency.

Method

Shoots from the eighth generation BA culture of Spiraea 'Froebelii' were subcultured on to a medium containing gibberellic acid (GA3) at 0, 0.5, 5.0, 10.0 or 50.0 mg 1-1, IBA at 0, 0.5, 5.0 or 10.0

mg l^{-1}, or 2iP at 0, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}. The medium was otherwise as described in Section 3.31 (contained BA). Shoot number, shoot length, explant growth and leaf length were recorded at the end of a four week culture period in light (16 hour photoperiod).

Results

Gibberellin

Shoot number decreased with GA concentration (Figure 265; Table 62). An analysis of variance (Table 63) showed that there was a significant effect on shoot formation due to gibberellin concentration ($p<.001$), BA concentration ($p<.001$) and a significant interaction between GA and BA concentration ($p<.001$).

Gibberellin promoted shoot elongation at BA concentrations of 0 to 5 mg l^{-1} (Figure 266), explant growth at concentrations of 0 to 2.5 mg l^{-1} (figure 267) and leaf length at BA concentrations of 0 to 0.5 mg l^{-1} (Figure 268). A significant effect due to GA concentration on shoot growth (Table 64) ($p<.001$) and a significant effect ($p<.01$) of GA concentration on explant growth (Table 65) were shown. Change in leaf

length with GA concentration was not significant (Table 66). Optimal GA concentrations were 5.0 mg l^{-1} for shoot elongation and 10 mg l for explant and leaf growth.

Auxin

Shoot number decreased with increase in IBA concentration (Figure 269; Table 67). A significant effect due to IBA concentration ($p<.001$), BA concentration ($p<.001$) and interaction between BA and IBA ($p<.001$) were recorded (Table 68).

Shoot length (Figure 270) and explant growth (Figure 271) increased with IBA concentration but no change in leaf length was recorded. These increases were significant ($p<.01$) for shoot length (Table 69) and for explant growth ($p<.05$) (Table 70).

2iP

A decrease in shoot number with increasing 2iP concentration was recorded (Figure 272; Table 71). An analysis of variance (Table 72) also showed a significant effect due to BA concentration ($p<.001$) and a significant interaction ($p<.001$) between BA and 2iP concentration.

Shoot length (Figure 273) and explant growth (Figure 274) increased after 2iP treatment. However,

these changes were not significant (Tables 73 and 74). No change in leaf size was recorded after 2iP treatment.

Table 62.

Mean shoot number after a 4 week incubation period on nutrient medium containing BA and GA : Spiraea 'Froebelii'.

BA mg l^{-1}	GA concentration (mg l^{-1})					
	0	0.5	5.0	10.0	50.0	Mean
0	1.25	1.0	0.5	0.25	0	0.6
0.1	22.5	18.5	11.25	7.25	4.5	12.8
0.5	17.5	13.25	9.5	6.5	4.75	10.3
1.0	14.0	9.25	6.25	5.25	4.0	7.75
2.5	14.0	12.25	10.75	8.0	3.75	9.75
5.0	11.5	10.25	8.75	5.0	3.25	7.75
10.0	7.25	7.0	6.75	4.25	1.75	5.4
Mean	12.57	10.21	7.68	5.21	3.14	7.76

L.S.D. = 1.79 (body of table).

L.S.D. = 0.675 (GA means).

L.S.D. = 0.798 (BA means).

Figure 265.
Mean shoot number formed on eighth generation explants after a four week incubation period on medium containing BA and GA.

Equation for curve $y = b_0 + b_1 \log_e x + b_2 \log_e x^2 + b_3 \log_e x^3$
$R^2 = .9789$
$r = -.9894$ ($p < .01$)

Figure 266.
Modal shoot length of ninth generation shoots after a four week incubation period on medium containing BA and GA.

Figure 267.
Explant growth (8th generation explant) in a four week culture period on medium containing BA and GA.

Figure 268.
Modal leaf length after a four week incubation period on medium containing BA and GA (9 th generation shoots).

Fig 265. Spiraea 'Froebelii'.

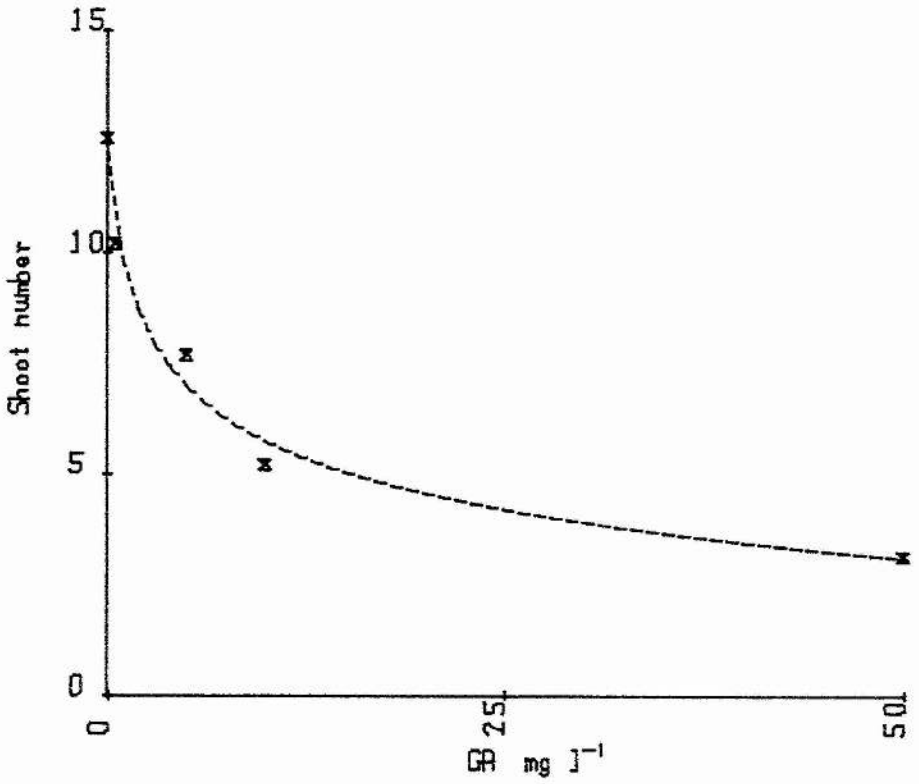


Fig 266. Spiraea 'Froebelii'.

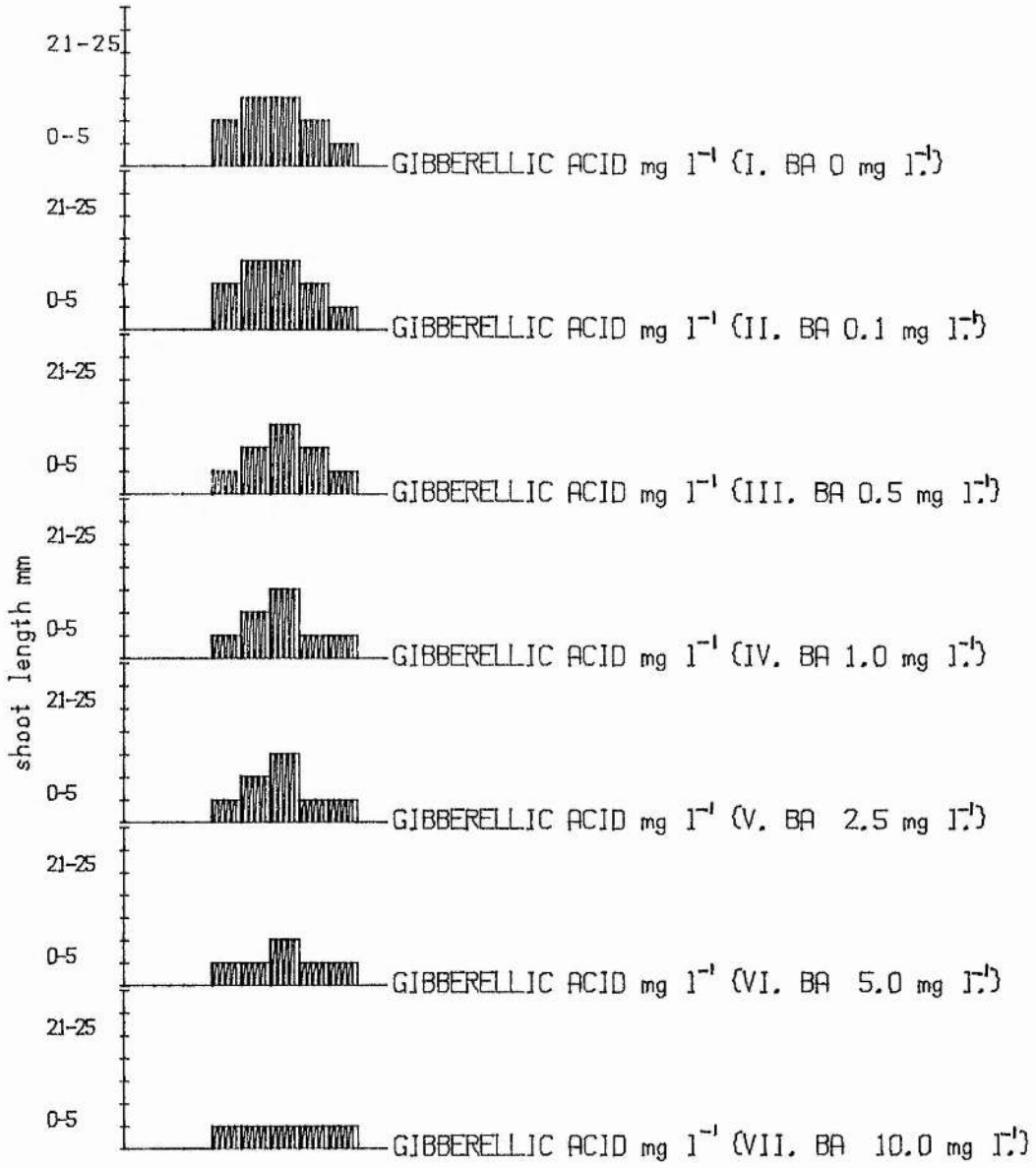


Fig 267. Spiraea 'Froebelii'.

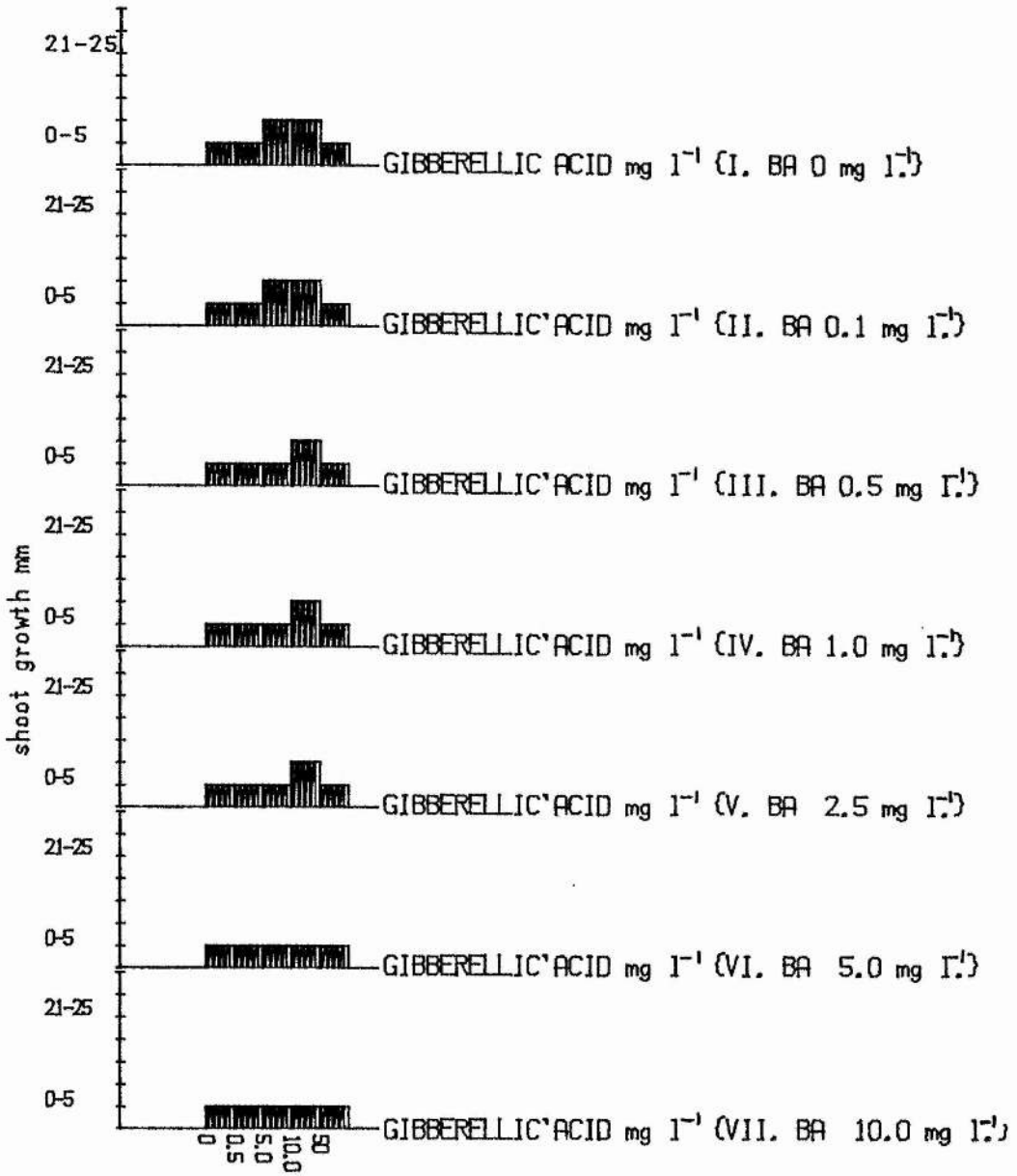


Fig 26. Spiraea 'Froebelii'.

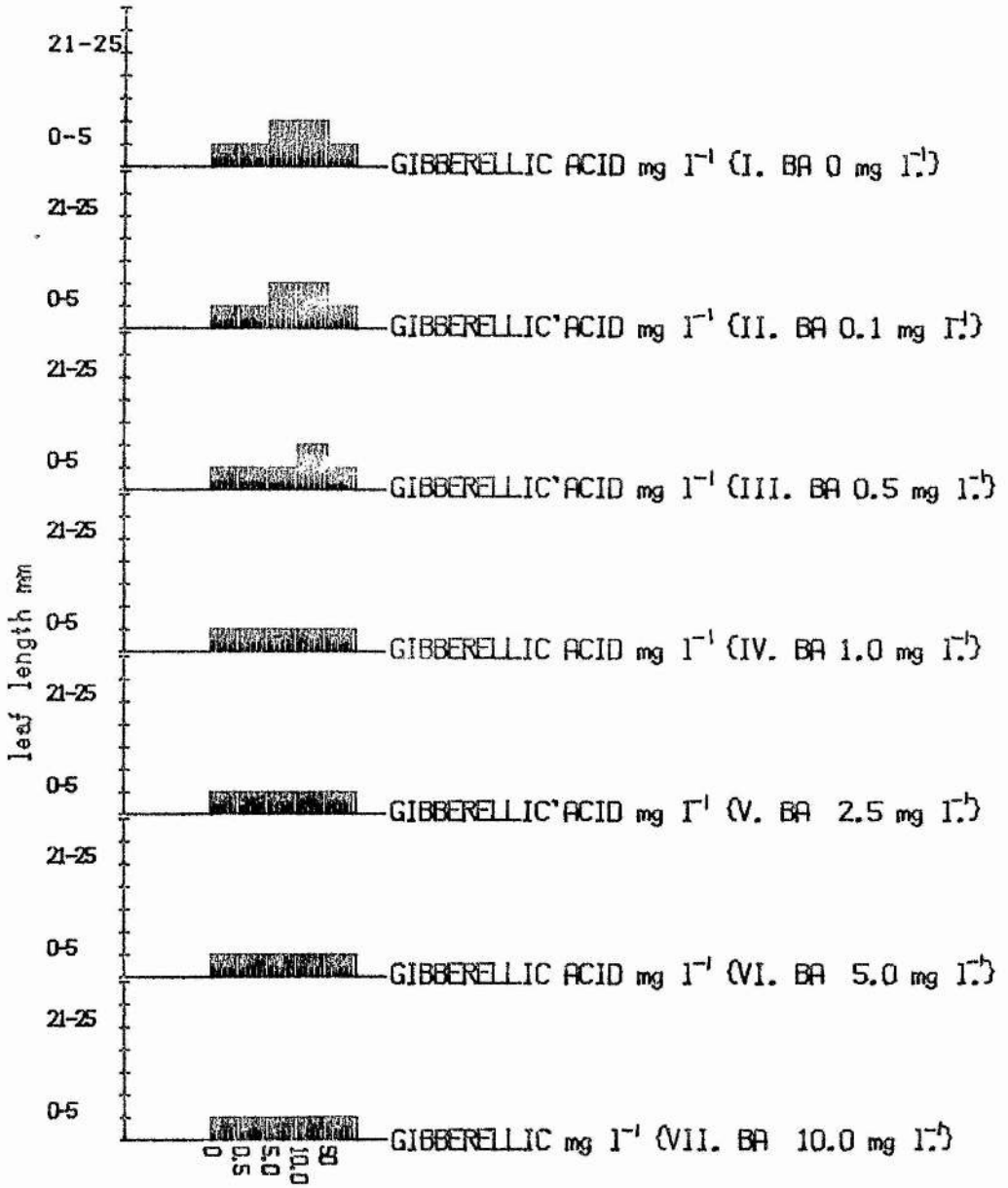


Table 63.

Analysis of variance for data presented in Table 62.

SOURCE	S.S.	D.F.	M.S.	F	p
GA conc	1595.400	4	398.850	261.337	<.001
BA conc	1852.971	6	308.829	202.353	<.001
Interaction	548.600	24	22.858	14.977	<.001
Error	160.250	105	1.526		
Total	4157.221	139			

Table 64.

Analysis of variance for data presented in Figure 266.

SOURCE	S.S.	D.F.	M.S.	F	p
GA conc	275.714	4	68.929	7.618	<.001
Error	271.429	30	9.048		
Total	547.143	34			

Table 65.

Analysis of variance for data presented in Figure 268.

SOURCE	S.S.	D.F.	M.S.	F	p
GA conc.	2184.098	3	728.033	6.700	<.01
Error	2607.879	24	108.662		
Total	4791.977	27			

(An arcsin $\sqrt{p}$ transformation was used).

Table 66.

Analysis of variance for data presented in Figure 268.

SOURCE	S.S.	D.F.	M.S.	F	p
GA conc.	880.159	3	293.386	2.455	N.S.
Error	2868.667	24	119.528		
Total	3748.826	27			

(an arcsin $\sqrt{p}$ transformation was used)

Figure 269.

Mean shoot number on eighth generation explants after a four week incubation period on medium containing BA and IBA.

Equation for curve $y = b_o + b_1 \log_e x + b_2 \log_e x^2$

$R^2 = .9766$

$r = -.9882$ ($p < .02$)

S.E. = 1.354

Figure 270.

Modal shoot length (9th generation shoots) after a four week incubation period on nutrient medium containing BA and IBA.

Figure 271.

Explant growth (from eighth generation) in a four week incubation period on medium containing BA and IBA.

Fig 260. Spiraea 'Froebelii'.

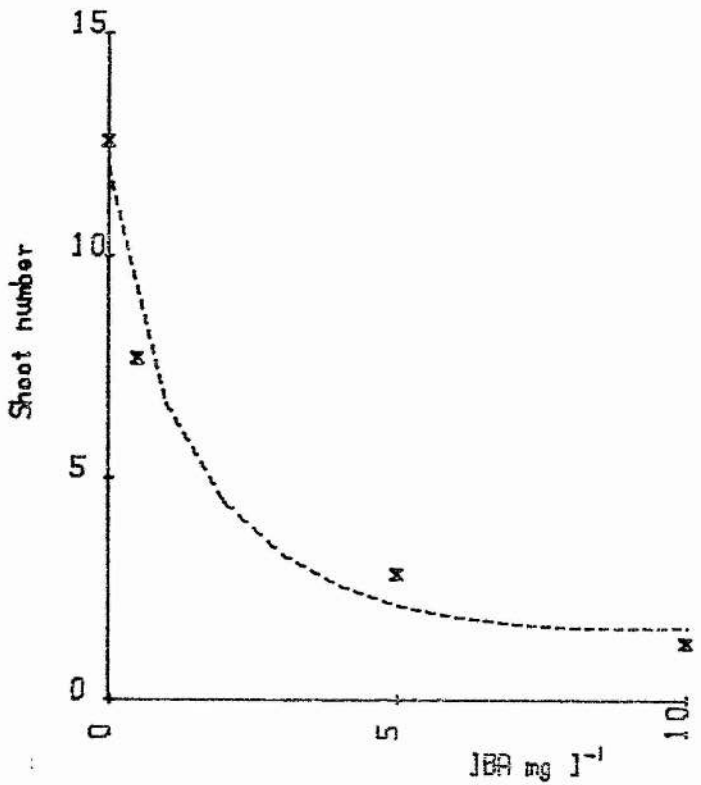


Fig 27a Spiraea 'Froebelii'.

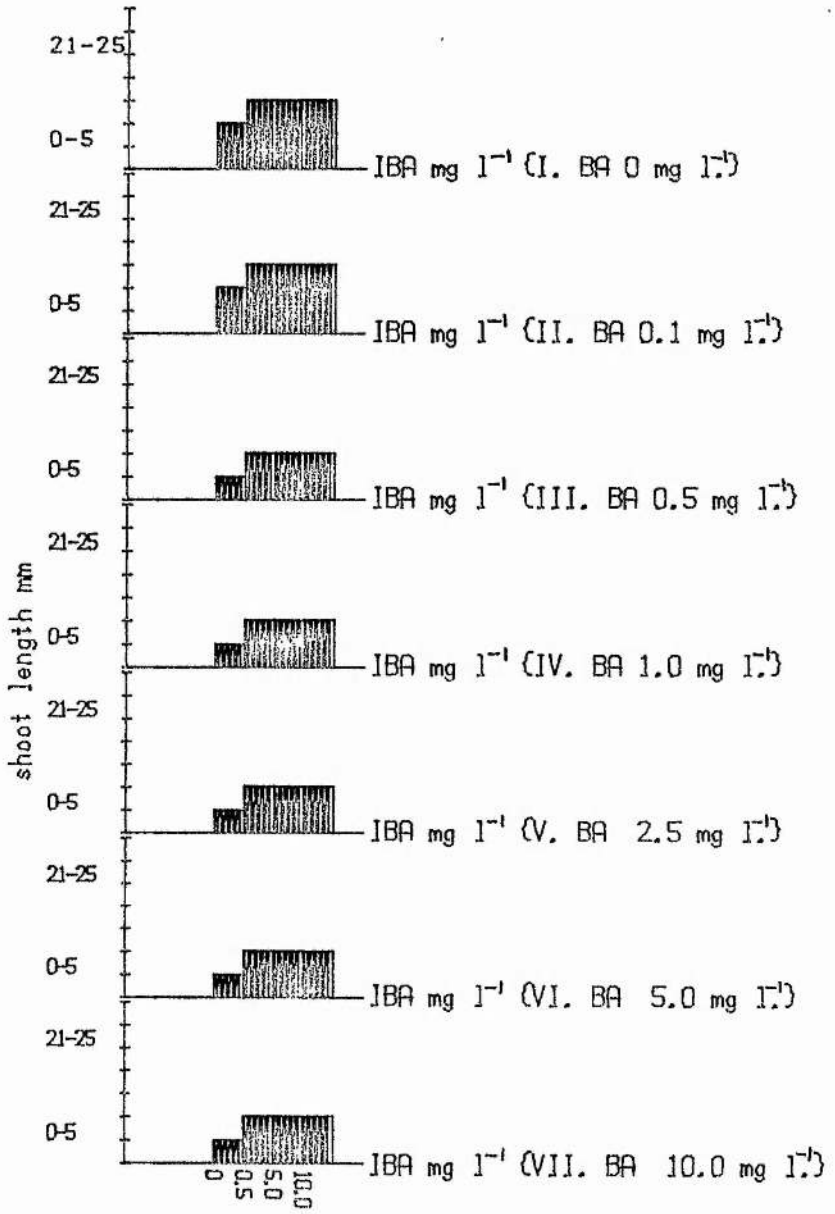


Fig 27. Spiraea 'Froebelii'.

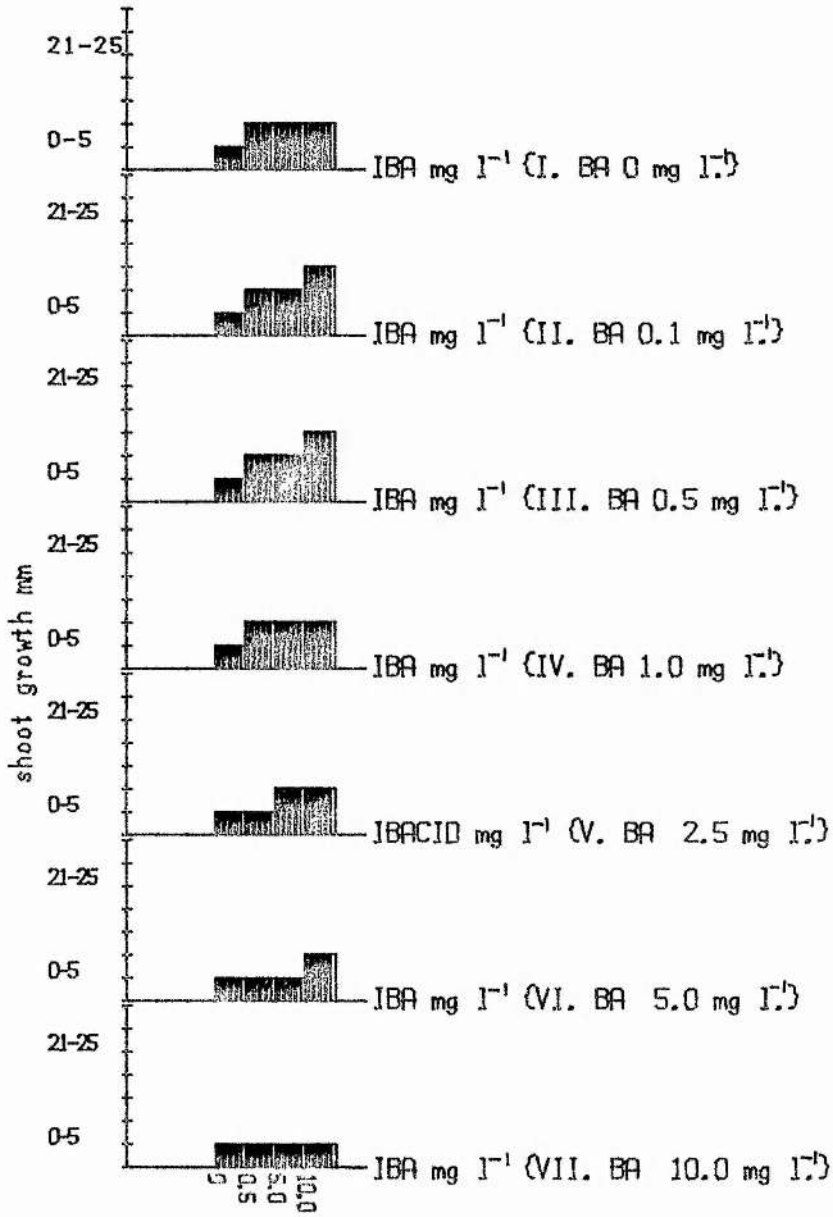


Table 67. Mean shoot number after a 4 week incubation period on nutrient medium containing BA and IBA: Spiraea 'Froebelii'.

BA mg l^{-1}	IBA concentration (mg l^{-1})				
	0	0.5	5.0	10.0	Mean
0	1.25	1.0	0.25	0	0.63
0.1	22.5	16.0	2.5	0.25	10.31
0.5	17.5	10.55	3.25	0.25	7.88
1.0	14.0	6.75	3.5	1.75	6.50
2.5	14.0	7.0	3.25	1.75	6.50
5.0	11.5	7.25	3.5	2.5	6.19
10.0	7.25	5.5	3.5	2.5	4.69
Mean	12.57	7.71	2.82	1.29	6.10

L.S.D. = 2.19 (body of table).

L.S.D. = 0.828 (IBA means).

L.S.D. = 1.096 (BA means).

Table 68. Analysis of variance for data presented in Table 67.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	2195.527	3	731.842	318.110	<.001
BA concentratio	851.1071	6	141.851	61.658	<.001
Interaction	862.036	18	47.891	20.817	<.001
Error	193.250	84	2.300		
Total	4101.920	111			

Table 69.

Analysis of variance for data presented in Figure 270.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	2253.200	3	751.067	6.927	<.01
Error	2602.186	24	108.424		
Total	4855.386	27			

(An arcsin $\sqrt{p}$ transformation was used).

Table 70.

Analysis of variance for data presented in Figure 271.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	3694.133	3	1231.378	4.333	<.05
Error	6821.087	24	284.212		
Total	10515.220	27			

(an arcsin$\sqrt{p}$ transformation was used)

Figure 272.

Mean shoot number at the end of a four week incubation period on medium containing BA and 2iP (9th generation shoots).

Equation for curve $y = b_0 + b_1x^1 + b_2x^2$

$R^2 = .9655$

$r = .9826$ ($p < .01$)

S.E. = 1.054

Figure 273.

Modal shoot length at the end of a four week incubation period on medium containing BA and 2iP - 9th generation shoots.

Figure 274.

Explant growth in a four week incubation period on medium containing BA and 2iP (Explant taken from 8th generation shoot culture).

Fig 272. Spiraea 'Froebelii'.

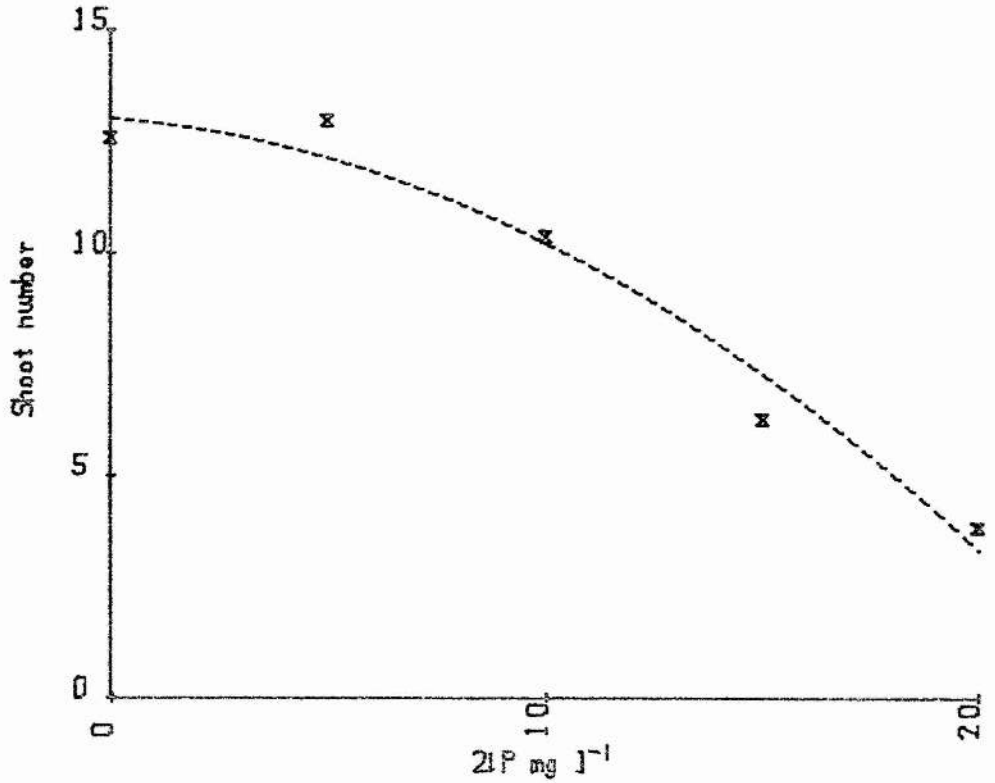


Fig 273. Spiraea 'Froebelii'.

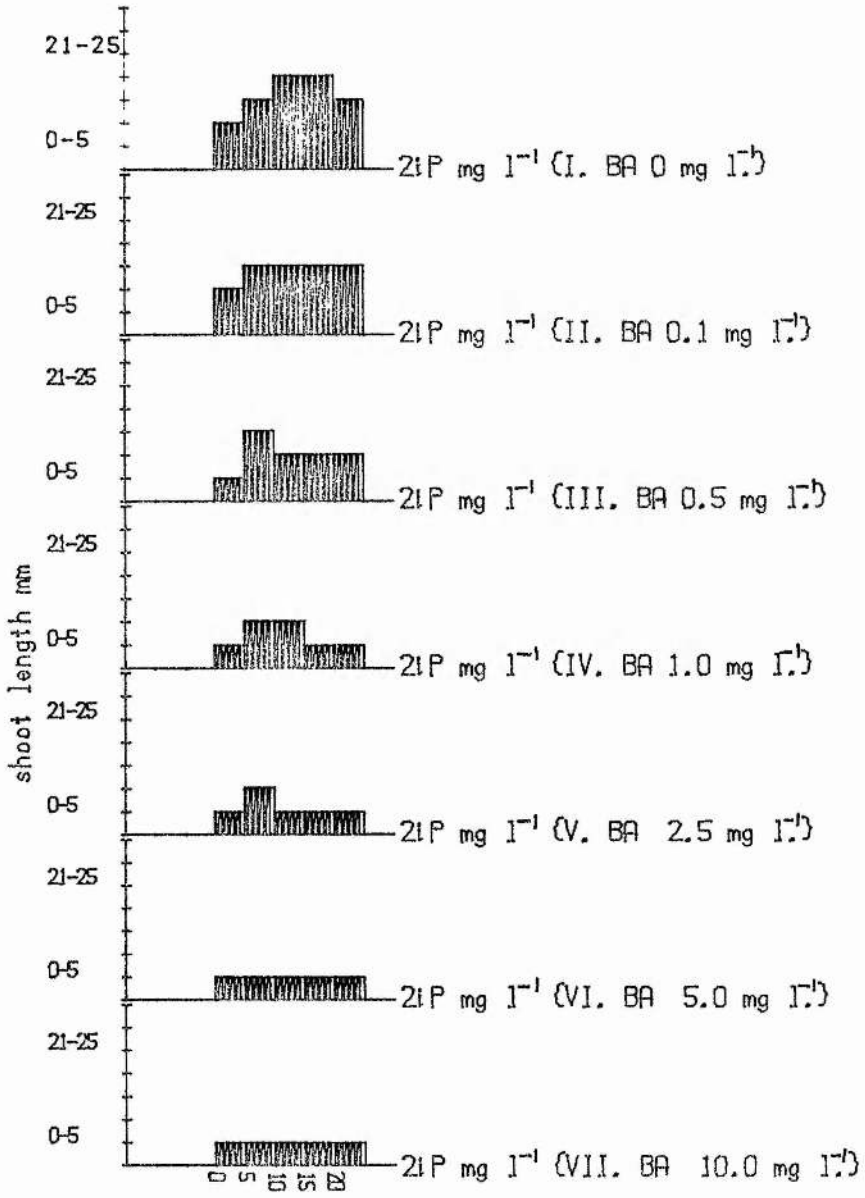


Fig 274. Spiraea 'Froebelii'.

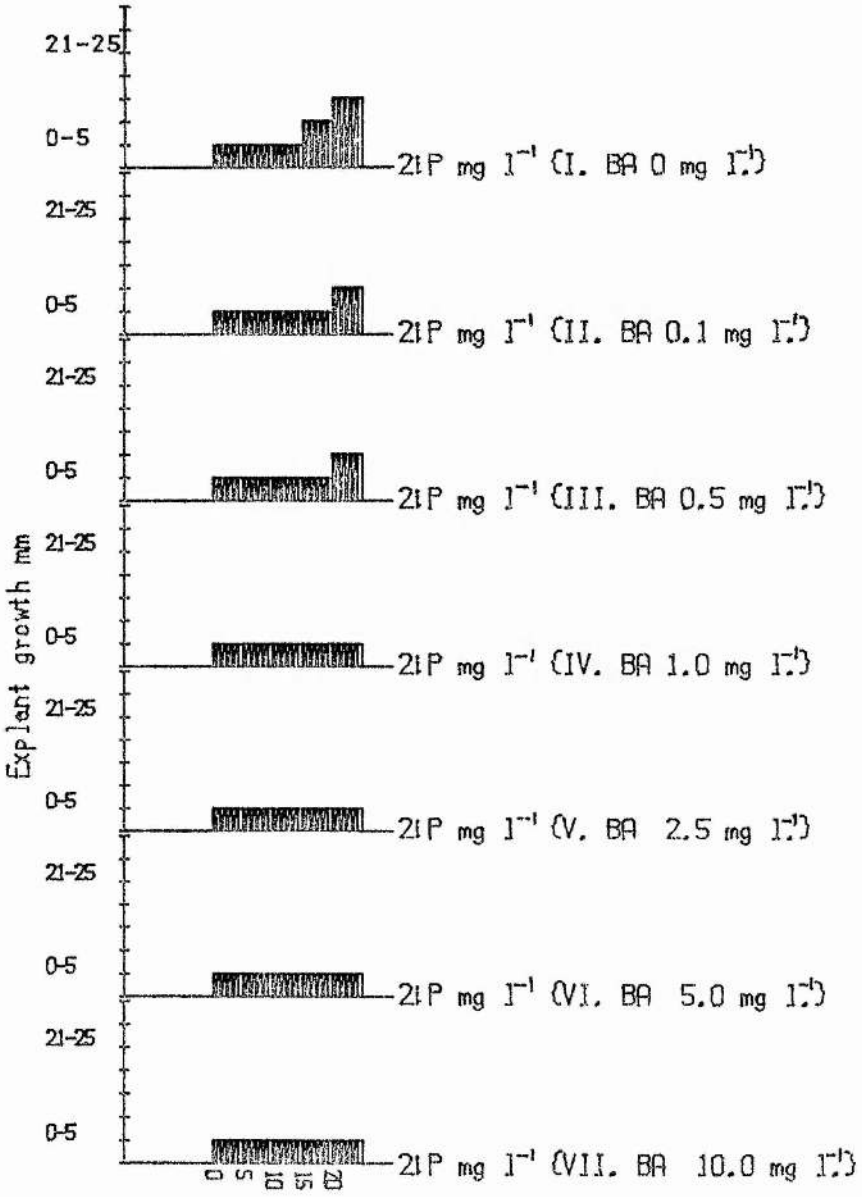


Table 71. Mean shoot number after a 4 week incubation period on nutrient medium containing BA and 2iP: Spiraea 'Froebelii'.

BA mg l^{-1}	2iP concentration (mg l^{-1})					
	0	5.0	10.0	15.0	20.0	Mean
0	1.25	10.5	15.75	8.5	6.53	8.50
0.1	22.5	24.25	15.5	10.0	4.5	15.35
0.5	17.5	19.0	15.5	8.75	4.5	13.05
1.0	14.0	15.75	12.75	7.25	4.0	10.75
2.5	14.0	11.75	6.5	4.75	3.75	8.15
5.0	11.5	6.75	4.25	3.0	2.5	5.60
10.0	7.25	2.75	2.25	1.5	1.0	2.95
Mean	12.57	12.96	10.36	6.25	3.82	9.19

L.S.D. = 2.26 (body of table).

L.S.D. = 0.853 (2iP means).

L.S.D. = 1.01 (BA means).

Table 72. Analysis of variance for data presented in Table 71.

SOURCE	S.S.	D.F.	M.S.	F	p
21P conc.	1806.186	4	451.546	185.024	<.001
BA conc.	2173.243	6	362.207	148.417	<.001
Interaction	1394.114	24	58.088	23.802	<.001
Error	256.250	105	2.440		
Total	5629.793	139			

Table 73.

Analysis of variance for data presented in Figure 273.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP conc.	744.977	4	186.244	0.776	N.S.
Error	7196.803	30	239.893		
Total	7941.780	34			

(An arcsin $\sqrt{p}$ transformation was used).

Table 74.

Analysis of variance for data presented in Figure 274.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP conc.	405.731	4	101.433	2.349	N.S.
Error	1727.607	40	43.190		
Total	2133.338	44			

(an arcsin $\sqrt{p}$ transformation was used)

3.33 SHOOT FORMATION UNDER CONDITIONS OF REDUCED ILLUMINATION

Section 3.31 showed that leaf size was decreased after repeated subculture of shoots on medium containing BA. The previous section (3.32) showed that exogenous growth regulators did not reverse this trend. Leaf morphology can vary with light intensity (Bidwell, 1979), small leaves being formed in leaves subjected to high illumination, and large leaves resulting at lower light intensities.

Reduction in total light received by the shoots can be achieved in two ways :- (1) by reduction of light intensity, and (2) by reduction in time of exposure to light (photoperiod). An experiment was conducted to determine whether reduction of leaf size is a change which can be reversed by reduction of light provided to the shoots.

Method

Shoots from the eighth generation culture of Spiraea 'Froebelii' were subcultured with the addition of BA as described in 3.31. Cultures were incubated under a range of light treatments as

follows :-

1. 16 hour photoperiod, 73 $\mu\varepsilon$ $m^{-2}s^{-1}$ (as in previous experiments)
2. 8 hour photoperiod, 73 $\mu\varepsilon$ $m^{-2}s^{-1}$
3. 16 hour photoperiod, 47 $\mu\varepsilon$ $m^{-2}s^{-1}$
4. 8 hour photoperiod, 47 $\mu\varepsilon$ $m^{-2}s^{-1}$

Reduced light intensity was obtained by using white gauze to shade the light.

Shoot number, shoot length, explant length and leaf length were recorded at the end of a four week culture period.

Results

Shoot number in different light treatments is given in Table 75. A significant effect due to illumination treatment ($p<.001$), BA concentration ($p<.001$) and a significant interaction between illumination treatment and BA concentration ($p<.001$) was recorded (Table 76). Most shoots were formed in illumination treatment (1). Reduction of light intensity and / or photoperiod resulted in reduced shoot number (Table 77).

Modal shoot length (Figure 275), initial explant growth (Figure 276) and leaf length (Table 80) were

increased by reduction of light intensity and / or photoperiod. A significant ($p<.001$) effect of illumination treatment on shoot length (Table 78) and explant growth (Table 79) was shown.

Table 75. Mean shoot number after a 4 week incubation period on nutrient medium containing BA : Change with reduced illumination : Spiraea 'Froebelii'.

BA mg l^{-1}	Illumination treatment (see text)				
	1	2	3	4	Mean
0	1.25	0.75	0.50	0	0.50
0.1	22.5	8.5	12.0	5.75	10.75
0.5	17.5	8.0	10.75	4.5	10.19
1.0	14.0	5.5	8.5	3.0	7.75
2.5	14.0	4.75	7.25	2.75	7.19
5.0	11.5	3.0	5.0	1.5	5.25
10.0	7.25	1.75	3.0	1.0	3.25
Mean	12.57	4.546	6.71	1.82	6.41

L.S.D. = 1.95 (body of table).

L.S.D. = 0.737 (illumination means).

L.S.D. = 0.975 (BA means).

Table 76. Analysis of variance for data presented in Table 75.

SOURCE	S.S.	D.F.	M.S.	F	p
Illumination	1753.464	3	584.488	320.895	<.001
BA conc.	1308.232	6	218.039	119.708	<.001
Interaction	538.411	18	29.912	16.422	<.001
Error	153.000	84	1.821		
Total	3753.107	111			

Table 77. Mean shoot number after a 4 week incubation period in 4 illumination treatments : Spiraea 'Froebelii'.

Illumination (see text)	Shoot number
1	12.57a
2	4.54c
3	6.71b
4	1.82d

Means followed by different letters are significantly different ($p<.05$).

Table 78. Analysis of variance for data presented in Figure 275.

SOURCE	S.S.	D.F.	M.S.	F	p
Illumination	2541.877	3	847.292	14.573	<.001
Error	1395.420	24	58.142		
Total	3937.296	27			

An arcsin $\sqrt{p}$ transformation was used.

Figure 275.
Modal shoot length (9th generation shoots) after a four week incubation period on medium containing BA : the effect of reduced illumination.

Figure 276.
Explant growth in a four week incubation period on medium containing BA : effect of reduced illumination.

Fig275. Spiraea 'Froebelii'.

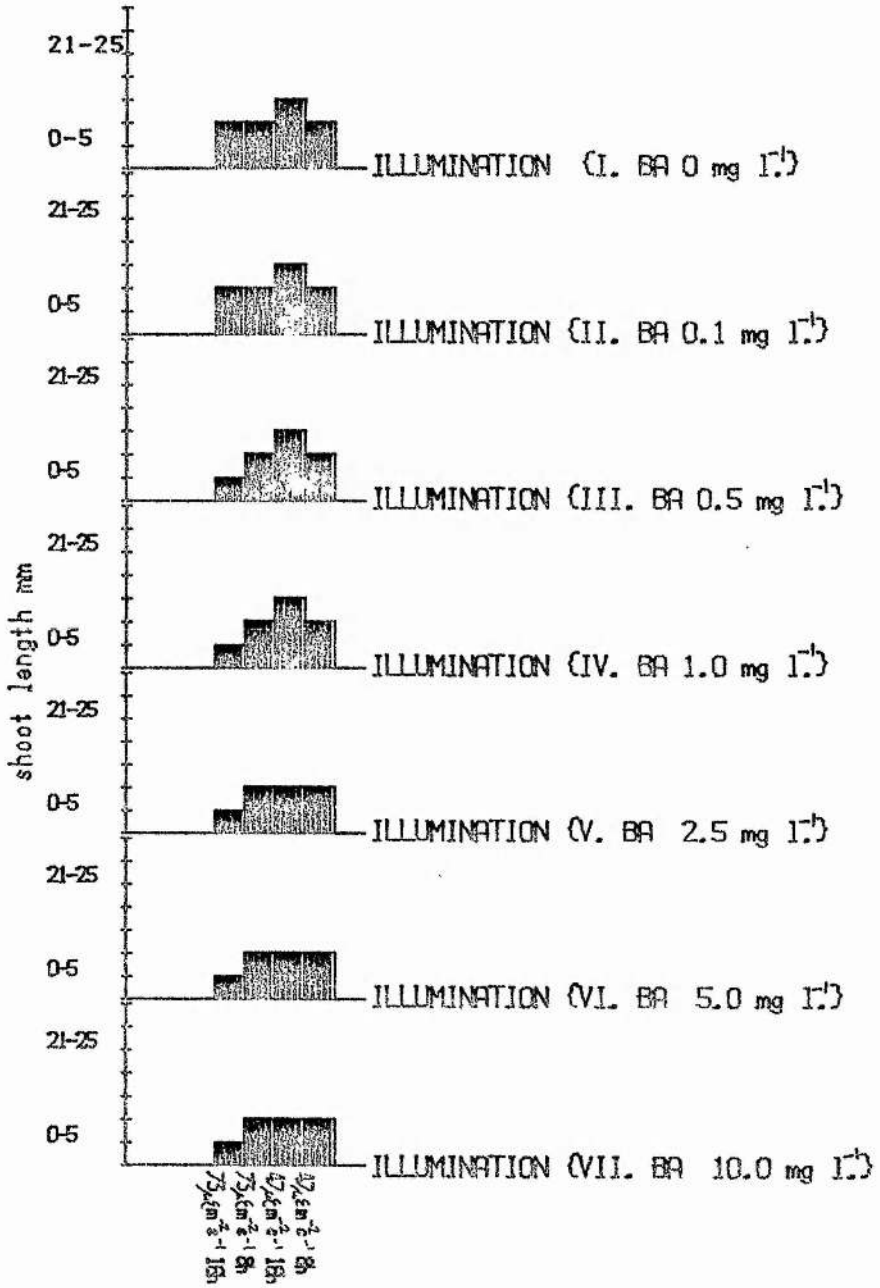


Fig 276. Spiraea 'Froebelii'.

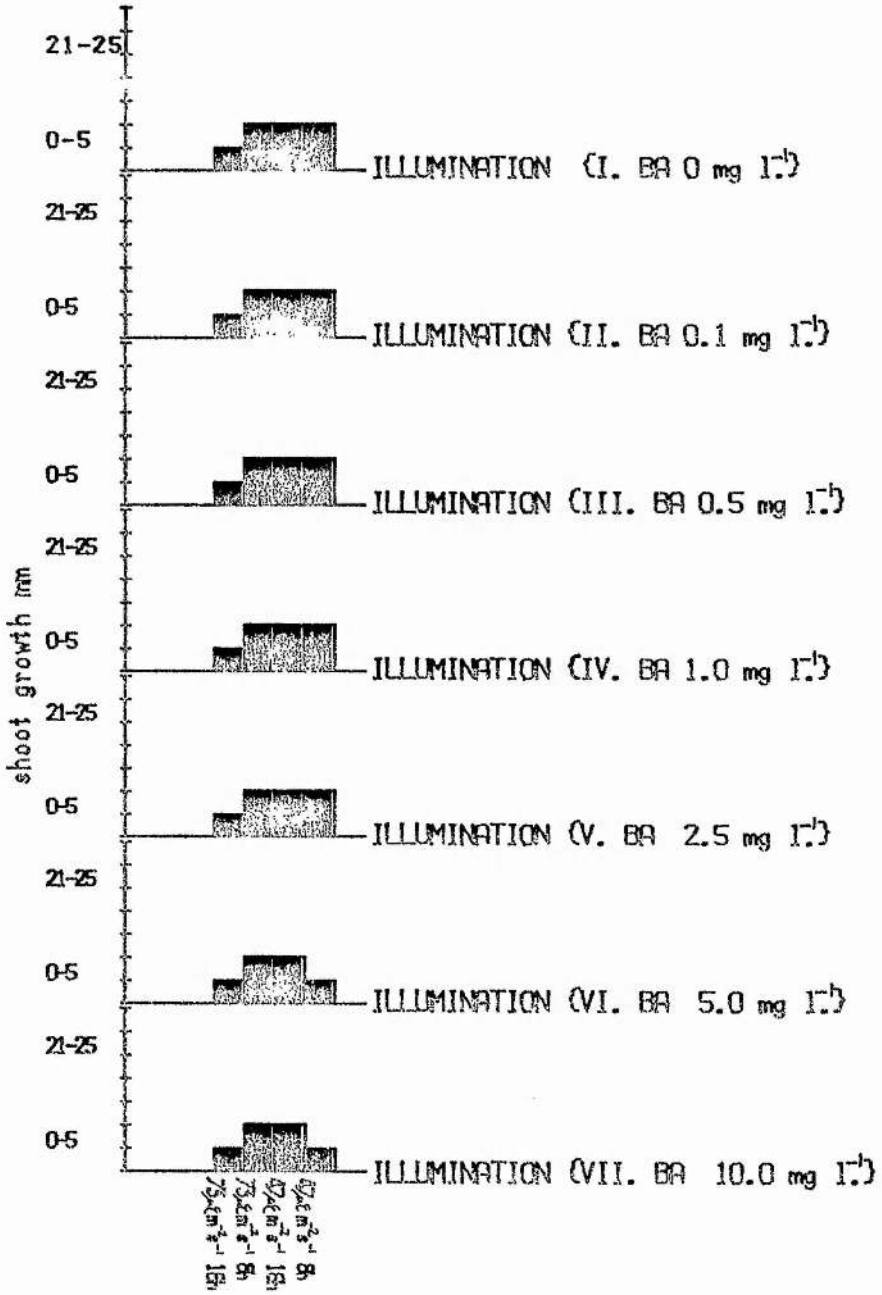


Table 79. Analysis of variance for data presented in Figure 276.

SOURCE	S.S.	D.F.	M.S.	F	p
Illumination	4270.401	3	1423.467	26.200	<.001
Error	1303.939	24	54.331		
Total	5574.341	27			

An arcsin $\sqrt{p}$ transformation was used.

Table 80. Modal leaf length (mm) at the end of a 4 week incubation period on medium containing BA : Reduced illumination treatment : Spiraea 'Froebelii'.

BA mg l^{-1}	Illumination treatment (see text)			
	1	2	3	4
0	0-5	6-10	6-10	6-10
0.1	0-5	6-10	6-10	6-10
0.5	0-5	6-10	6-10	6-10
1.0	0-5	6-10	6-10	6-10
2.5	0-5	6-10	6-10	6-10
5.0	0-5	6-10	6-10	6-10
10.0	0-5	6-10	6-10	6-10

3.34 DISCUSSION

An increase in shoot formation was observed in the first one to three generations depending on species. Initial increase in caulogenesis in the first propagative generations suggests reduction of an inhibitor or change in growth regulator status. Differentiation in callus occurs more readily after transfers of callus tissues to fresh media for incubation (Avetisov, 1976; Saunders and Bingham, 1975) and it was suggested that a preculture period may be necessary to eliminate inhibitory substances (unspecified) from callus. Section 3.2 is supportive of this theory i.e. that substances inhibitory to shoot initiation are present in the shoot apex and the axillary buds. It is therefore suggested, if this is an inhibitor response, that reduction or inactivation of the inhibitor results from subculture of new shoots. Long term culture could account for these changes by binding, leaching or other destructive processes. Alternatively, a change in source : sink relationships could account for these phenomena.

A decline in shoot morphogenesis was observed beyond a certain threshold for each species. In

Chaenomeles, the decline in shoot morphogenesis only occurred at a BA concentration of 5 mg l^{-1}. This species only formed axillary shoots (not adventitious shoots - see below) and this may show that axillary shoots are more stable in culture than adventitious shoots as noted by Murashige (1977c). The decline in shoot morphogenesis was paralleled by a decrease in shoot length, reduction of leaf size and an increase in number of callus cells. Experiments were conducted to determine whether these morphological changes are related to reduced caulogenesis.

If an inhibitory substance is present, as suggested by Avetisov (1976), then its presence may promote shoot formation at a low concentration and inhibit it at a high concentration. The fact that parallel reduction of shoot elongation was observed suggests that auxin or gibberellin could be this substance. Both shoot form and leaf development are controlled in other species by gibberellin / cytokinin ratios. BA / GA ratios control the formation of leaves or spines in Opuntia (Mauseth and Halperin, 1975); and the form of shoots induced from callus is determined by GA / CK ratio in the medium - high ratios gave tall thin shoots with narrow leaves, while

low ratios gave short shoots with rounded leaves (Engelke et al., 1973).

Auxin or gibberellin additions to eighth generation shoots should reverse this trend if the hypothesis is true i.e. it is hypothesised that if an increase in shoot initiation occurred as a result of these treatments, then auxin or gibberellin depletion could be the cause of the decline in shoot initiation. However, although shoot length increased after auxin treatment, and shoot and leaf length increased after gibberellin application, shoot initiation was decreased. This experiment suggests that neither gibberellin nor auxin limits shoot initiation. Rugini and Verma (1983) found that GA did not increase elongation of subcultured shoots of Prunus but the concentrations which they used were lower (0.1 to 1.0 mg l^{-1}) than those found to be effective in my experiment (5 mg l^{-1}).

A rise in endogenous auxin content after cytokinin application has been reported (Imaseki et al., 1975). It is possible, therefore, that auxin concentration could gradually increase with number of subcultures. This could account for production of more callus cells in later cultures. Auxin strongly promotes callus formation (Chapter 5), especially in the presence of

cytokinin. However, if auxin and cytokinin control the pattern of differentiation, then some questions arise. If endogenous auxin content increases after BA application, then do shoots differentiate on supplemental application of BA? This did not occur. It is unlikely that auxin is present at concentrations high enough to inhibit shoot initiation. This is supported by the experiment described above where exogenous auxin increased shoot length. If considerable quantities of auxin were synthesised by the tissue, then the expected shoot length would be greater than observed.

In Section 3.1, it was demonstrated that longer shoots were formed when the cytokinin supplied was 2iP. Additionally, this section demonstrated that 2iP did not have the same effect on decline of shoot morphogenesis as did BA. Thus, BA enhanced decline in the test species (Spiraea). An experiment was conducted to determine whether 2iP could reverse this BA-induced decline, but, in eighth generation cultures shoot initiation was not promoted by 2iP. The role of ethene in the stunting of cytokinin-treated shoots was discussed in Section 3.14. If cytokinin promotes ethene synthesis (shown in Chapter 8), then increasing

the cytokinin concentration would not reverse the observed reduction in shoot length.

Leaf shape can vary with light intensity (Bidwell, 1979). It is suggested that reduction in leaf size could result from high light intensity. An experiment in reduced light intensity and / or daylength showed that leaf size and shoot length were increased by reduction in illumination. Shoot initiation was decreased by decreased illumination and / or decreased length of photoperiod, i.e. by the total light received by shoots.

In summary, lack of auxin and/or gibberellin, combined with high light intensity for long photoperiod, may account for the observed reduction in shoot length and leaf size. These morphological changes are phenotypic and are reversible as shown. However, these factors are probably not involved in the control of shoot initiation which declines predictably but irreversibly with repeated subculture.

This apparently irreversible decline in shoot formation was observed in shoots repeatedly cultured on media containing BA and on media containing 2iP. From these results, it is not possible to deduce whether the presence of cytokinin causes this decline in shoot

formation. Additional experimentation is necessary to determine whether a decline in caulogenic potential occurs in the absence of cytokinin.

Repeated subculture of shoots is frequently practiced in commercial micropropagation. Although some propagators have noticed changes after long-term culture, these have not been reported in the literature. Swartz et al. (1983) found no increase in phenotypic instability up to the seventh proliferation culture in blackberry, and Rugini and Verma (1983) cultured Prunus shoots (reduced in size) for two years on medium containing BA and NAA. However, propagators are agreed that it is 'not advisable' to repeatedly subculture shoots for more than three or four generations (Murashige, 1977b; 1977c).

It has been noted that an increased number of abnormalities occur in plants maintained in constant environmental conditions of long photoperiod and relatively high temperature for long periods of time (Ticknor, unpub.); and other workers have reported a reduction in growth (Lanphear and Meahl, 1961) or even spring dormancy (Whalley, 1977) in the season following a winter of prolonged vegetative growth induced by daylength increase. This suggests that some variation

in either photoperiod or temperature may be essential for maintainance of normal growth of plants. This would be provided by natural seasonal variation. In my experiments, constant conditions were maintained throughout, and therefore isolated shoots might show adverse effects from deprivation of normal seasonal fluctuations. This hypothesis is supported by the fact that reduction of daylength and light intensity increased shoot length and leaf size. Bulbs developed in vitro must be chilled to ensure their successful transfer to soil (Murashige, 1977c), but season-mimicking alterations of temperature are not normally given during the course of repeated subcultures (Murashige, 1977c).

The first generation of shoots was cultured in May and the period of observed decline occurred from November to February. The decline could be due to lack of a seasonal change. Reduction in caulogenesis in autumn was largely overcome after one subculture transfer (see Section 3.2). Thus a seasonal effect is unlikely to be important in culture.

Callus and cell suspensions, cultured for a long period of time, undergo changes in their potential for differentiation (Torrey, 1967, Smith and Street, 1974).

Torrey (1967) observed a progressive loss in organogenetic capacity with increasing polyploidy and aneuploidy in long term callus cultures of Pisum; and in tobacco callus (Murashige and Nakano, 1967), the capacity for shoot formation was found to be severely reduced in callus strains with extensive aneuploidy. It was concluded that reduction in differentiation was a consequence of karyotype changes (Thomas et al., 1979).

However, Sacristan and Melchers (1969) regenerated extreme aneuploid plants from 20 year old tobacco tumor tissues; Sacristan and Lutz (1970) showed that differentiation could take place in aneuploid tobacco cultures; Gould (1978) found that morphogenic potential was lost without any change in the karyotype of cultured cells of Brachycome; and Wochok and Wetherell (1972) reported that addition of kinetin could restore decline in regenerative response in long-term callus cultures. These results indicate that decline in morphogenic potential may not always be a result of genetic change, but rather, can be a reversible change.

However, other changes such as the change observed in my experiments, do not seem to be so simply reversible. Larkin and Scowcroft (1981) concluded that

frequency of variation in callus cultures is too great to be accounted for by a chemical mutagen component of the medium. Such changes may therefore be the result of other types of genetic change.

After prolonged growth on medium containing maltose as the carbon source, the cells of a soybean suspension culture had lost one third of their ribosomal genes (Jackson, 1980). A change was evident within two generations. Such changes may also occur in other species. Other types of change could include chromosome rearrangement and transposition of genetic elements.

Chromosome rearrangement can result in loss of genetic material which may result in phenotypic variants. For example, a rearrangement may delete or otherwise switch off a dominant allele allowing the recessive allele to affect the phenotype. This has been referred to as culture-induced hemizygosity (Siminovitch, 1976). Transposable genetic elements, stretches of DNA which can move from one locus in the genome to another (Calos and Miller, 1980) could play a role in variation in cultures.

Heritable changes can be induced in flax when grown in different nutrient environments (Durrant,

1962; 1971). Varying nitrogen, phosphorus and potassium supply resulted in stable genotrophs differing in height, nuclear DNA content (Evans et al., 1966), ribosomal DNA (Cullis, 1975; 1976) and isozyme band pattern (Cullis and Kolodynska, 1975; Cullis, 1981). Cullis (1981) discussed the mechanism for ribosomal DNA change and suggested two possibilities - (1) via an extrachromosomal intermediate or (2) by unequal mitotic recombination followed by cell selection. Changes in peroxidase enzymes in flax may be caused by a DNA rearrangement, possibly mediated by a transposable element (Cullis, 1977; 1979) but such changes are always in one direction and this may indicate a deletion of material at or near the site of rearrangement (Cullis, 1981).

Change in potential for morphogenesis in shoot cultures was a gradual repeated change. This indicates that exposure to some element in the culture environment (possibly cytokinin) induces a slight change every time the shoots are exposed to a renewed supply of the element. This suggests that repeated deletions of genetic material could be occurring due to fluxes in exogenous, cytokinin, nutrient or sucrose.

3.4 CAULOGENESIS IN CALLUS

Cells of differentiated tissue normally dedifferentiate before differentiated cells can form from them. Callus is relatively undifferentiated tissue and so, only one process, differentiation, may be involved in shoot formation. It is therefore probable that shoots will form from callus more readily and rapidly than from differentiated tissue. Experiments were conducted to investigate this and to determine if exogenous growth regulator requirements are the same for shoot formation from callus as for differentiation from shoot explants.

In Chapter 5, it is shown that callus varies in growth rate and appearance with growth regulator application. Some callus cultures grew rapidly in the absence of exogenous growth regulators or when supplied with low levels of auxin, whereas others were dependent on an exogenous growth regulator source and grew much more slowly. The potential of these different types of callus to regenerate shoots was compared.

Section 3.3 showed that shoot formation increased with shoot subculture in the first one to

three subculture periods. An experiment was conducted to determine whether shoot formation from callus followed the same pattern.

3.41 SHOOT FORMATION FROM CALLUS

Method

Experiment 1.

10 mm (diameter) callus explants of Rhododendron concinnum was used in this experiment. Three types of callus formed in experiments detailed in Chapter 5, were selected :- 1) lumpy friable callus, 2) glistening white smooth callus, and 3) green callus (see Section 5.1 for additional descriptions and for details of growth regulators previously applied). BA or 2iP was incorporated in the nutrient medium at the following concentrations :- BA - 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 or 40.0 mg l^{-1} ; 2iP - 0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 or 60.0 mg l^{-1} . Nutrient medium was either solid (gelled with agar) or liquid (with callus supported on a filter paper bridge). Agar has been shown to adversely affect callus growth (see Section 2.23) and therefore was

eliminated from part of this experiment.

Incubation was in light (16 hour photoperiod). Cultures were examined for shoot formation each week for 12 weeks.

Experiment 2.

Callus derived from three different growth regulator treatments (supplied for callus initiation - see Section 5.1) were used. These were 1) IBA, 2) BA and 3) 2iP. Chaenomeles japonica was selected for experimentation because callus formation was promoted by both auxin and cytokinin.

BA and 2iP were incorporated in the nutrient medium at the concentrations detailed in Experiment 1 above. Cultures were incubated in light (16 hour photoperiod) and were examined for shoot formation each week for 12 weeks.

Experiment 3.

Callus of types (1) and (2), detailed in Experiment 1 above, of Rhododendron concinnum was subcultured six times to medium identical to that which had induced the initiation of the callus and in identical environmental conditions (see Section 5.1). Callus from the second, fourth and sixth subculture was transferred to medium containing BA at the

concentrations specified for Experiment 1 above. Incubation was in light (16 hour photoperiod). Cultures were inspected for shoot formation every week for 12 weeks.

Results

Experiment 1.

No green shoots were formed in any treatment. However, callus of type 1 (lumpy friable callus) formed small projections in liquid culture at BA concentrations 5.0, 10.0 and 15.0 mg l^{-1} after 9 weeks of incubation (Plate 13). These projections failed to develop any further. The projections were examined microscopically and it was determined that some vascular tissue had differentiated.

Experiment 2.

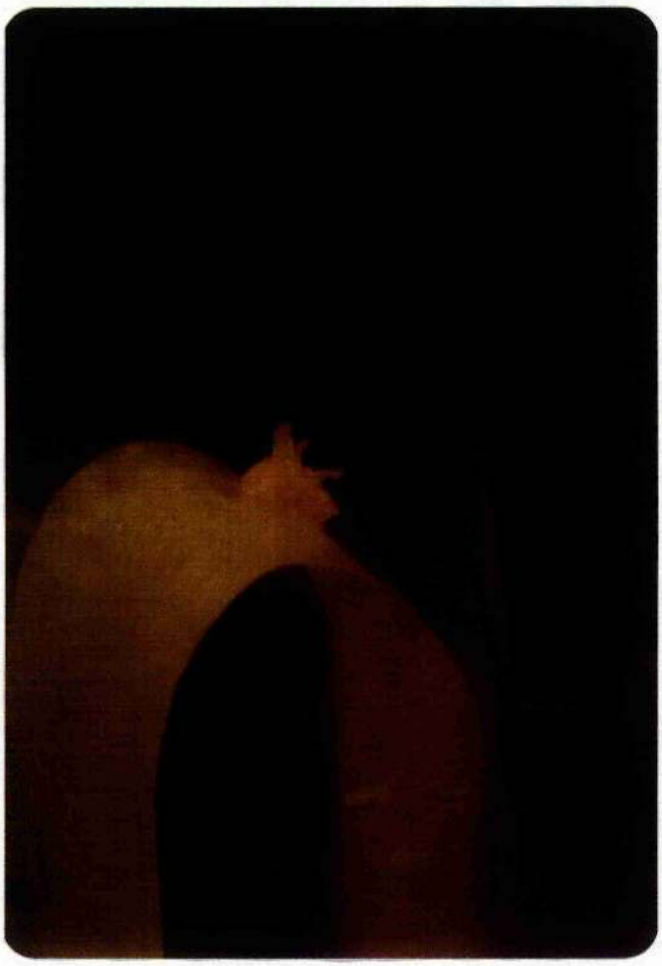
One shoot formed when BA was provided at 5.0 mg l^{-1} in one culture only after 8 weeks of incubation. No other shoots formed in any treatment.

Experiment 3.

No shoots were formed in any treatment in either type of callus tested.

Plate 13.

Projections formed on callus of Rhododendron concinnum in response to exogenous cytokinin.



3.42 DISCUSSION

The hypothesis that shoots would form more readily from callus than from stem tissue was disproved. Only one true shoot formed and this formed at a fairly high cytokinin concentration and took longer (8 weeks) to form than shoots initiated from stem tissue (2 to 3 weeks - see 3.1). Similarly, the projections which formed in Experiment (1) required high BA concentrations to develop (and differentiate vascular tissue) and differentiation took a long time (9 weeks).

Shoot or bud formation from callus occurs readily in many herbaceous species , for example, in tobacco (Skoog and Miller, 1957; Thorpe and Meier, 1972; Brossard, 1976), but occurs less frequently in callus of woody plants (Narayanaswamy, 1977). In Populus, shoots were initiated from callus by the addition of BA at 0.14 mg l^{-1} but higher BA concentrations inhibited shoot formation (Wolter, 1968). This conflicts with my results which indicate that a high BA concentration is probably required for shoot formation in Rosaceous and Ericaceous plants.

Other workers have found that two media transfers with appropriate incubation times are frequently

required before shoot or bud formation will occur. This has been demonstrated in callus of Arabidopsis (Avetisov, 1976) and in alfalfa (Saunders and Bingham, 1975). It was suggested by these authors that the initial culture period may be necessary to eliminate inhibitory substances including auxins. In support of this, Section 3.1 demonstrated a rise in differentiation of shoots from shoot explants over the first 1 to 3 subculture periods. However, such a rise was not found when callus was the starting material (Experiment 3).

The non-formation of shoots may be due to endogenous growth regulator status of the callus. Tobacco callus produces GA, the endogenous content being affected by culture conditions including exogenous cytokinin concentration (Lance et al., 1976). If the callus used here synthesised GA in response to cytokinin treatment, shoots might not form (GA inhibits formation of bud-producing meristematic nodules - Murashige, 1964). Cytokinin treatment may also cause a rise in auxin synthesis (see Section 3.1) and this also could inhibit shoot formation. Alternatively, shoot formation may be inhibited by the exogenous growth regulator used to initiate the callus from shoot

explants. 2,4-D was used to initiate callus of Type (2) (smooth glistening white callus). 2,4-D can cause chromosomal aberrations and suppress organ differentiation (Sheridan, 1975). In callus of cereals, embryonic callus formed on medium containing 2,4-D when supplied in combination with IAA and kinetin, but not on medium containing only 2,4-D (Nabors et al., 1983).

The state of division of the callus cells at the time of cytokinin application may also be important in determining shoot formation. In tobacco callus, organogenesis could not be induced in the early stages of culture, but in the later part of the same subculture (stationary phase and later) organogenesis was enhanced (Noriko, 1972). In my experiments, cytokinin was incorporated in the nutrient medium and therefore was supplied at the beginning of the culture period. Noriko's work suggests that if cytokinin was supplied later in the culture period, shoots might form more readily.

IV. RHIZOGENESIS

4.1 AUXIN ACTIVITY IN ROOT FORMATION

This section examines the relationship between auxin concentration and rhizogenesis to determine whether exogenous auxin is essential for root initiation and whether control of root initiation can be affected by auxin concentration.

Excised shoots are examined in relation to their potential for adventitious root formation both in a controlled greenhouse environment and *in vitro*. These environments were used to determine whether difference in response is due to auxin application or environment.

Rooting was also studied in 'mini-cuttings' (shoots excised after one *in vitro* culture period on medium containing BA) to determine if these shoots differ from previously uncultured shoots in rooting potential i.e. whether BA pretreatment or *in vitro* culture conditions have an effect on rooting under greenhouse conditions.

Intact plants were examined for their potential to initiate adventitious roots to answer the question - do adventitious organs only form on excised plant parts?

4.11 ROOT FORMATION IN STEM CUTTINGS AND INTACT PLANTS

Method

See Section 2.3 for details of selection and preparation of cuttings, auxin preparations, rooting medium and greenhouse environmental conditions.

Experiment 1.

Stem cuttings of the following species were used in this experiment :-

Ericaceae: *Arctostaphylos media*, *Arctostaphylos uva-ursi*, *Erica carnea*, *Gaultheria hispidula*, *Kalmia angustifolia*, *Rhododendron arboreum*, *R. chamae-thomsonii*, *R.* 'Chikor', *R.* 'Chinsayii', *R. dauricum*, *R. fastigiatum*, *R. forrestii*, *R. keiskei*, *R. leucaspis*, *R. lutescens*, *R.* 'P.J.M. Victor', *R. racemosum*, *R.* 'Vuyk's rosy red', *R. williamsianum*, *Vaccinium vitis-idaea*.

Rosaceae:- *Chaenomeles japonica*, *Cotoneaster dammeri*, *Crataegus brachyacantha*, *Crataegus* 'Toba', *Malus* 'Dainty', *Malus* 'Golden Hornet', *Potentilla* 'Coronation Triumph', *Potentilla*

'Sutter's Gold', Prunus cerasifera, Prunus tomentosa, Pyracantha coccinea, Spiraea 'Froebelii'.

Cuttings were 1) not treated with auxin or 2) given a quick dip (IBA 0.3% for 5 seconds). There were 25 replicate cuttings in each treatment for each species.

Number of rooted cuttings was recorded after 4 weeks.

Experiment 2.

Stem cuttings of the following species were used in this experiment:- Arctostaphylos uva-ursi, Rhododendron 'PJM Victor', Prunus cerasifera, Spiraea 'Froebelii'.

Cuttings were treated with IBA as a quick dip at the following concentrations :- 0, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%. Root number was recorded after 4 weeks.

Experiment 3.

Species used in this experiment were the same as for Experiment 2 above. Cuttings (25 replicates) were either cuttings from whole plants (macro-cuttings), shoots derived from one four week

in vitro culture period on BA medium (mini-cuttings 1) or shoots derived from BA sprays of whole plants (see Section 3.1)(from treatment which gave maximal shoot number).

Macro-cuttings were treated with 1) no auxin or cytokinin, 2) IBA (0.3%), 3) IBA (0.3%) + BA (0.2%, or 4) IBA (0.3%) + BA (0.4%). Mini-cuttings were given treatments 1) and 2) only. The above treatments were given as a quick dip.

Number of rooted cuttings was recorded after 1, 2, 3, 4, 5, 6, 7 and 8 weeks.

Experiment 4.

Intact plants (one year old) of Arctostaphylos uva-ursi and Spiraea 'Froebelii' were sprayed twice per week until run-off (aerial portion of the plant) with one of the following treatments :-

1. 300 ppm IBA
2. 300 ppm IBA + 1% DMSO
3. distilled water
4. distilled water + 1% DMSO

Plants were examined for adventitious root formation after 4 weeks.

Experiment 5.

Methodology was as described for Experiment 4. One spray only of auxin + DMSO was given. IBA concentrations were 0, 100, 200, 300, 400, 500 and 1000 ppm. Plants were examined for adventitious root formation after 4 weeks.

Results

Experiment 1.

Percentage of cuttings which had rooted after 4 weeks is given in Table 81 for Rosaceae and Table 82 for Ericaceae. Analyses of variance (Tables 83 and 84) showed a significant effect ($p<.05$) due to IBA treatment, species and a significant interaction between species and IBA treatment. Auxin pretreatment enhanced rooting in 6 of the 12 species of Rosaceae and in 9 species of Ericaceae.

Experiment 2.

Fitted curves for root number against IBA concentration were plotted (significance of fit - $p<.001$ in each case) (Figures 277 to 280). The curves were quartic plots of the form

$y = b + b_1 x + b_2 x^2 + b_3 x^3 + b_4 x^4$

Optimal values for IBA concentration and root number were calculated from these models. These are given in Table 85.

An analysis of variance (Table 86) showed a significant effect due to IBA concentration ($p<.001$), a significant effect due to species ($p<.001$) and a significant interaction ($p<.001$) between these. It was shown (Table 87 - table of means) that the order of effectivness of IBA concentration when calculated over all four species was 1) 0.2%, 2) 0, 0.1%, 0.3%, 3) 0.4%, 4) 0.5% ($p<.05$). Most roots were formed in Spiraea followed by Prunus, then Arctostaphylos and Rhododendron formed fewest roots.

Experiment 3.

Percentage of cuttings rooted after 1, 2, 3, 4, 5, 6, 7 and 8 weeks is given in Figures 281 to 312 (fitted curves - $p<.001$).

Rate of rooting was calculated as

$$\sum_{i=1}^{n} y / f * w * 7$$

where n = number of weeks

f = final root count

w = week of interval

i.e. proportional to the maximum possible root number. If all rooted on the first day then unity would result.

There was a significant effect due to treatment, species and a significant treatment interaction ($p<.001$) (Table 88). Table 89 shows that the order of species for rate of rooting when calculated across all treatments was (1) Spiraea, (2) Arctostaphylos, (3) Prunus and (4) Rhododendron ($p<.05$). Macro-cuttings rooted significantly more slowly than mini-cuttings ($p<.05$) (Table 90). IBA significantly speeded up rooting in mini-cuttings ($p<.05$) and macro-cuttings ($p<.05$). Adding BA to IBA in macro-cuttings had no effect on rate of rooting ($p<.05$).

A few mini-cuttings did not survive (Table 91). Significantly more mini-cuttings died in treatments which included IBA than in treatments where IBA was absent ($p<.001$) (Table 82). No macro-cuttings died in the course of the experiment.

Experiment 4.

No adventitious roots formed in any plant or treatment.

Experiment 5.

No adventitious roots formed in any plant.

Experiment 6.

No preformed root initials or primordia for possible future adventitious shoot or root production were observed in either macro or mini cuttings.

Table 81.

Percentage of cuttings rooted after 4 weeks : Rosaceae.

Species	% rooted
Chaenomeles japonica	95a
Cotoneaster dammeri	60b
Crataegus brachyacantha	100a
Crataegus 'Toba'	48c
Potenti lla 'Coronation Triumph'	100a
Potentilla 'Sutter's Gold'	95a
Prunus cerasifera	63b
Prunus tomentosa	56bc
Pyracantha coccinea	37d
Spiraea 'Froebelii'	100a

Probabilities based on arcsin $\sqrt{p}$ transformation ($p<.05$).

Table 82.

Percentage of cuttings rooted after 4 weeks : Ericaceae.

Species	% rooting
Rhododendron fastigiatum	100
Rhododendron chamae-thomsonii	93
Vaccinium vitis-idaea	86
Rhododendron keiskei	79
Rhododendron chinsayii	76
Rhododendron racemosum	73
Rhododendron 'chikor'	71
Kalmia angustifolia	70
Gaultheria hispidula	56
Rhododendron 'vuyk's'	52
Arctostaphylos uva-ursi	50
Rhododendron dauricum	49
Rhododendron 'P.J.M. Victor'	49
Erica carnea	49
Rhododendron lutescens	48
Rhododendron leucaspis	34
Rhododendron forrestii	26
Arctostaphylos media	25
Rhododendron arboreum	16
Rhododendron williamsianum	11

probability based on arcsin $\sqrt{p}$ transformation ($p<.05$)

Table 83.

Analysis of variance for data presented in Table 81.

SOURCE	S.S.	D.F.	M.S.	F	p
+/- IBA	28.800	1	28.800	6.227	<.05
Species	2971.050	9	330.117	71.377	<.001
Interaction	310.450	9	34.494	7.458	<.001
Error	277.500	60	4.625		
Total	3587.800	79			

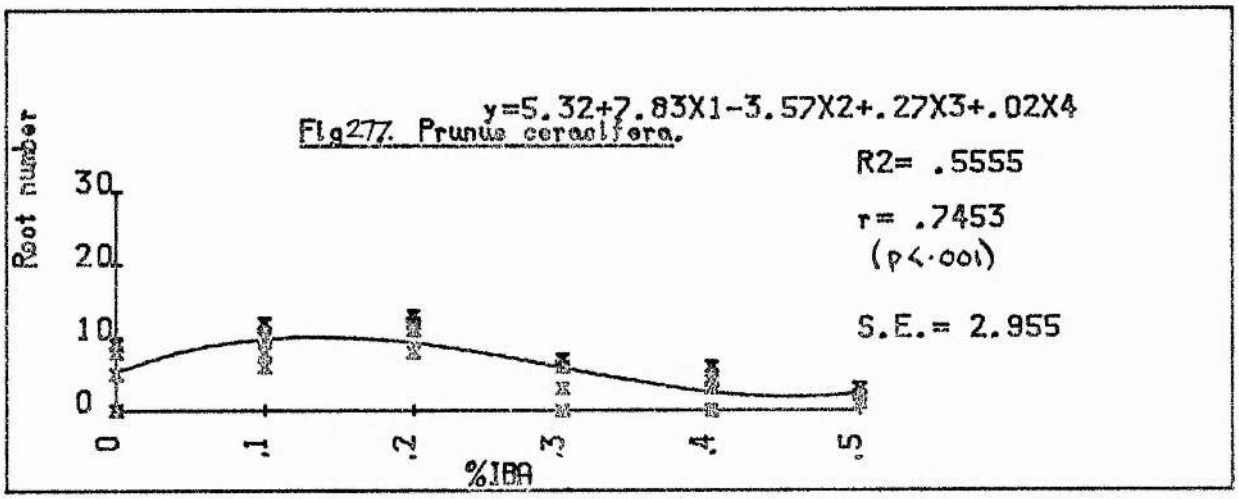
Table 84.

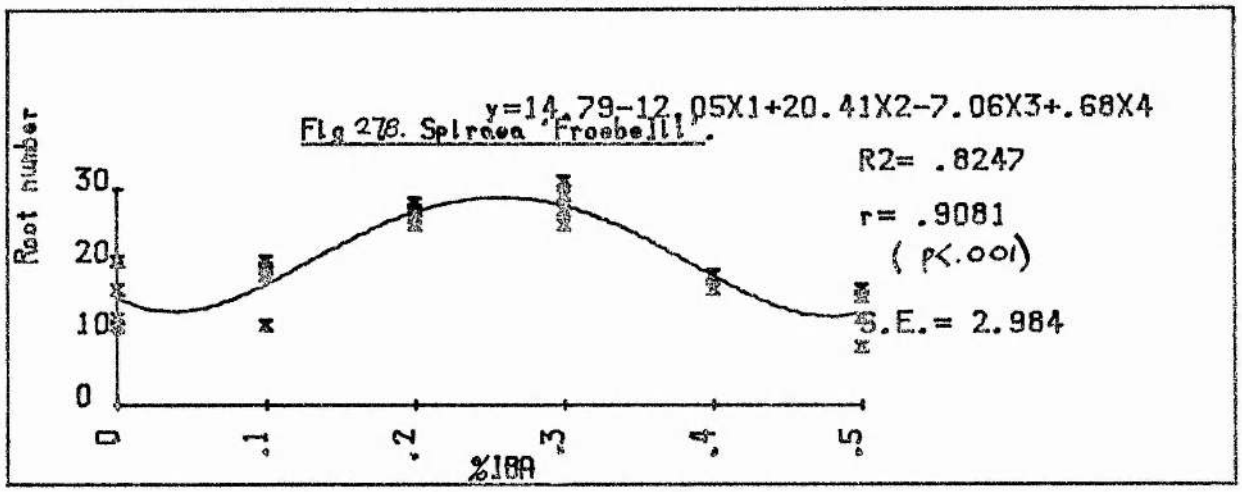
Analysis of variance for data presented in Table 82.

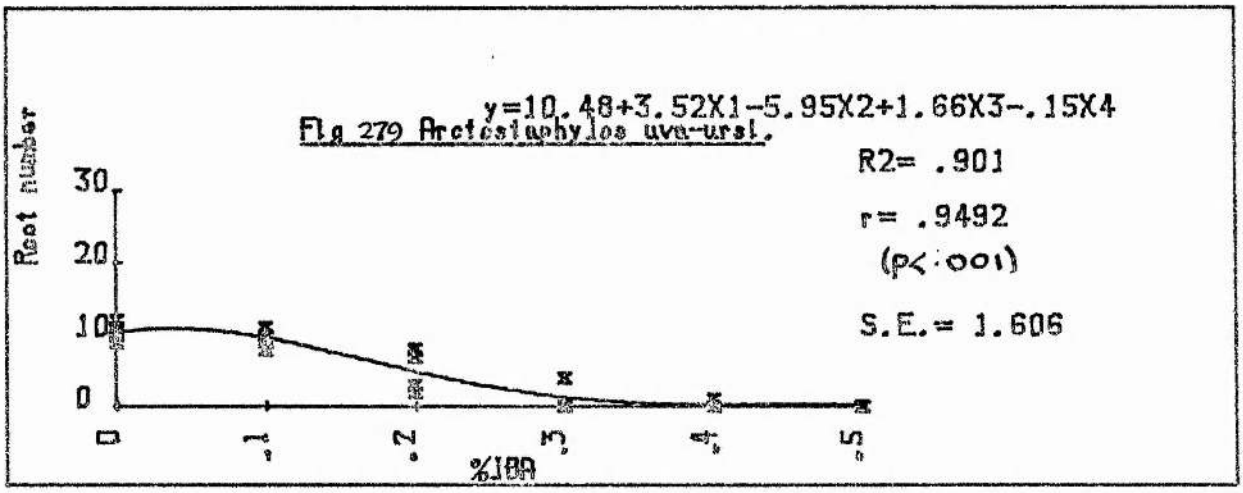
SOURCE	S.S.	D.F.	M.S.	F	p
+/- IBA	400.056	1	400.056	23.127	<.001
Species	4338.119	19	228.322	13.199	<.001
Interaction	3881.819	19	204.306	11.811	<.001
Error	2075.750	120	17.298		
Total	10695.744	159			

Figures 277 to 280.

Number of roots formed on stem cuttings after treatment with IBA (recorded after 4 weeks).


y=5.32+7.83X1-3.57X2+.27X3+.02X4
Fig 277. Prunus cerasifera.
R2= .5555
r= .7453
(p<.001)
S.E.= 2.955
Root number
30
20
10
0
0
.1
.2
.3
.4
.5
%IBA


y=14.79-12.05X1+20.41X2-7.06X3+.68X4
Fig 278. Spiraea 'Froebelli'.
R2= .8247
r= .9081
(p<.001)
S.E.= 2.984
Root number
30
20
10
0
0
.1
.2
.3
.4
.5
%IBA


y=10.48+3.52X1-5.95X2+1.66X3-.15X4
Fig 279 Arctostaphylos uva-ursi.
R2= .901
r= .9492
(p<.001)
S.E.= 1.606
Root number
30
20
10
0
0
.1
.2
.3
.4
.5
%IBA

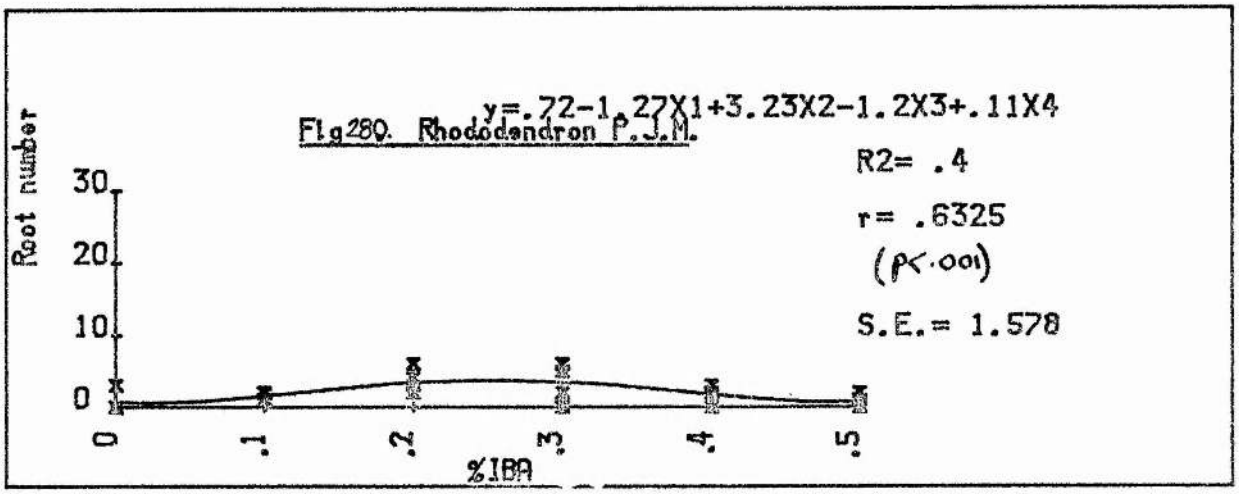

y=.72-1.27X1+3.23X2-1.2X3+.11X4
Fig 280. Rhododendron P.J.M.
R2= .4
r= .6325
(P<.001)
S.E.= 1.578
Root number
30
20
10
0
0
.1
.2
.3
.4
.5
%IBA

Table 85.

Optimal values for IBA concentration and root number from fitted curves of the form:-

$y=b+b_1 x^1+b_2 x^2+b_3 x^3+b_4 x^4$.

Species	Optimal IBA	Max root no.
Prunus cerasifera	0.13%	10.14
Spiraea 'Froebelii'	0.26%	28.89
Arctostaphylos uva-ursi	0.03%	11.05
Rhododendron 'P.J.M. Victor'	0.25%	3.80

Table 86.

Analysis of variance for data given in Figures 277 to 280.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	594.958	5	118.992	21.553	<.001
Species	4431.208	3	1477.069	267.545	<.001
Interaction	921.292	15	61.419	11.125	<.001
Error	397.500	72	5.521		
Total	6344.958	95			

Table 87. Mean root number after IBA treatment.

Species	IBA concentration (mg l^{-1})						
	0	0.1	0.2	0.3	0.4	0.5	Mean
Prunus cerasifera	5.5	9.0	11.0	4.0	3.25	2.25	5.83
Spiraea 'Froebelii'	14.75	17.0	26.5	28.0	17.5	12.75	19.42
Arctostaphylos uva-ursi	10.5	9.5	5.0	1.0	0.25	0	4.38
Rhododendron 'P.J.M. Victo	0.75	1.5	3.75	3.25	1.75	0.75	1.96
Mean	7.88	9.25	11.56	9.06	5.69	3.94	7.90

L.S.D. = 3.32 (body of table).

L.S.D. = 1.66 (IBA means).

L.S.D. = 1.36 (Variety means).

Figures 281 to 312.

Number of roots formed during an eight week period on macro and mini cuttings.

Figures 281 to 284. Prunus cerasifera - macro cuttings
Figures 285 to 286. Prunus cerasifera - mini cuttings (1)
Figures 287 to 288. Prunus cerasifera _mini cuttings (2)
Figures 289 to 292. Spiraea 'Froebelii' - macro cuttings
Figures 293 to 296. Arctostaphylos uva-ursi - macro cuttings
Figures 297 to 298. Arctostaphylos uva-ursi - mini cuttings(1)
Figures 299 to 300. Arctostaphylos uva-ursi - mini cuttings(2)
Figures 301 to 302. Spiraea 'Froebelii'- mini cuttings (1)
Figures 303 to 304. Spiraea 'Froebelii' - mini cuttings (2)
Figures 305 to 308. Rhododendron 'PJM Victor' - macro cuttings
Figures 309 to 310. Rhododendron 'PJM Victor'-mini cuttings(1)
Figures 311 to 312. Rhododendron 'PJM Victor'-mini cuttings(2)

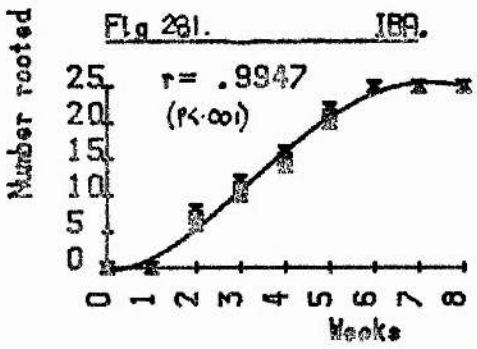


Fig 281. IBA.
r= .9947
(P<.001)
Number rooted
Weeks

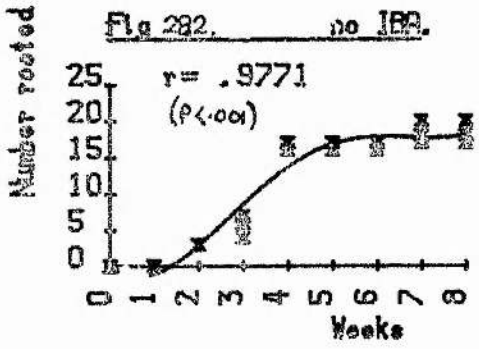


Fig 282. no IBA.
r= .9771
(P<.001)
Number rooted
Weeks

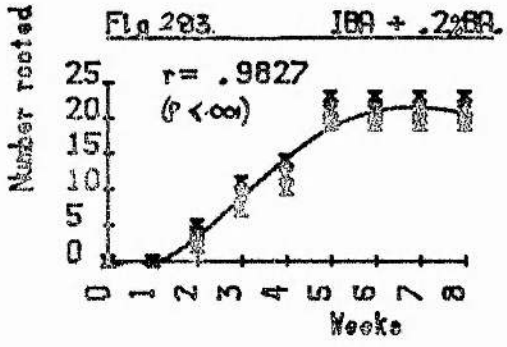


Fig 283. IBA + .2%BA.
r= .9827
(P<.001)
Number rooted
Weeks

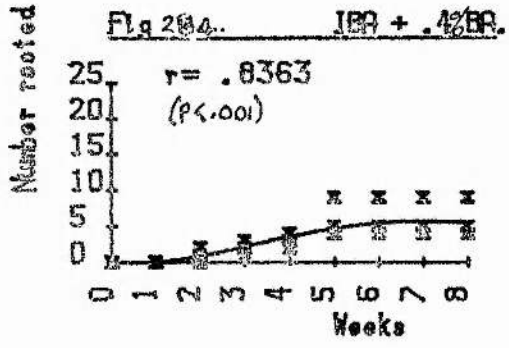


Fig 284. IBA + .4%BA.
r= .8363
(P<.001)
Number rooted
Weeks

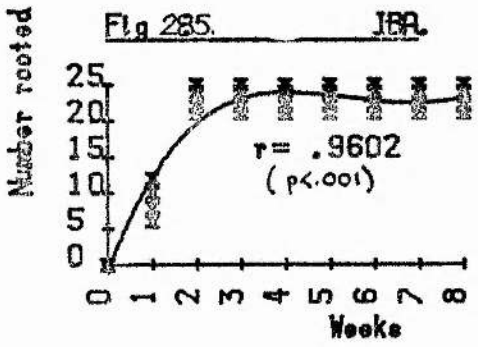


Fig 285. IBA.
Number rooted
25
20
15
10
5
0
r = .9602
(p<.001)
0 1 2 3 4 5 6 7 8
Weeks

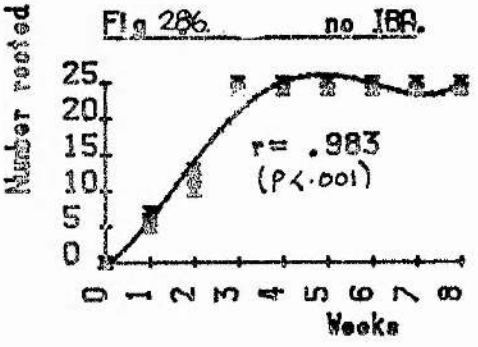


Fig 286. no IBA.
Number rooted
25
20
15
10
5
0
r = .983
(p<.001)
0 1 2 3 4 5 6 7 8
Weeks

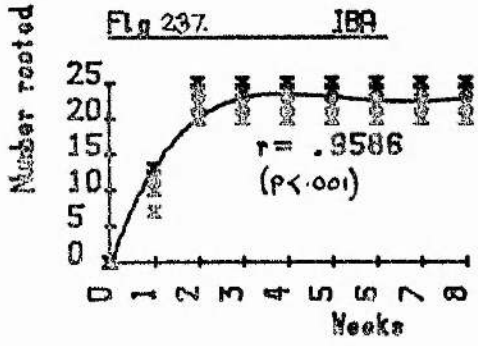


Fig 287. IBA
Number rooted
25
20
15
10
5
0
r = .9586
(p<.001)
0 1 2 3 4 5 6 7 8
Weeks

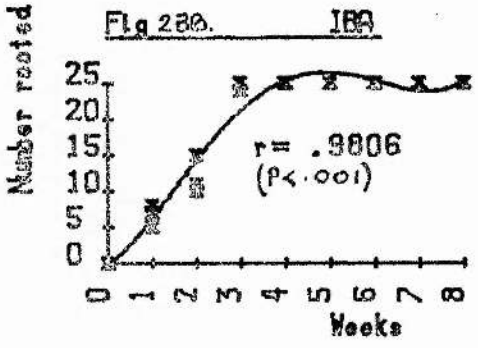


Fig 288. IBA
Number rooted
25
20
15
10
5
0
r = .9806
(p<.001)
0 1 2 3 4 5 6 7 8
Weeks

Fig 289. 0.3% IBA.

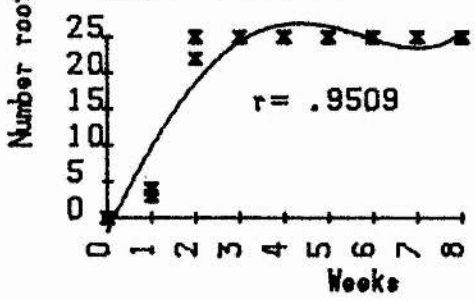


Fig 290. No IBA.

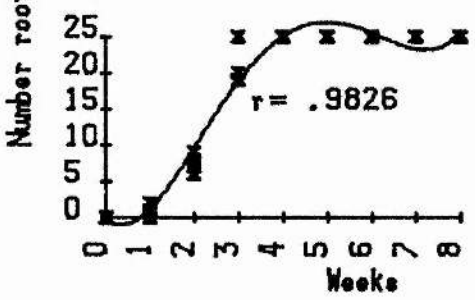


Fig 291. 0.3%IBA + 0.2%BA.

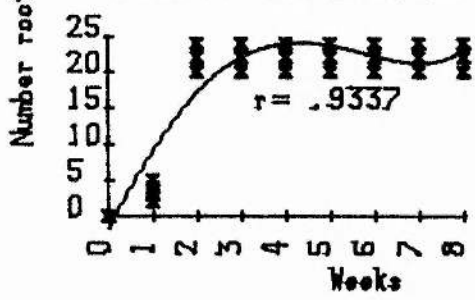


Fig 292. 0.3% IBA + 0.4%BA.

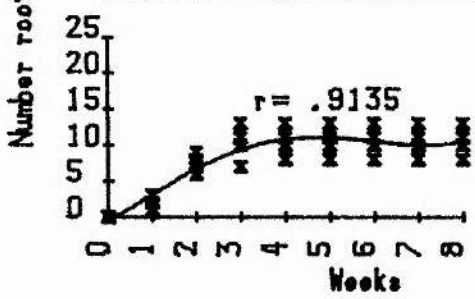


Fig 293. 0.3% IBA.

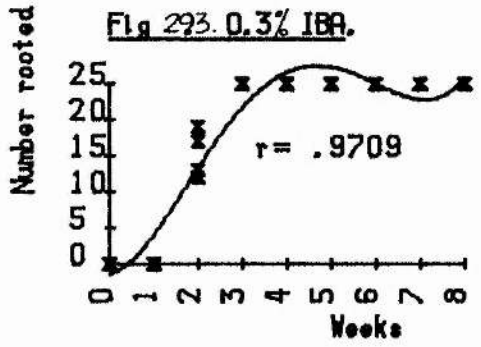


Fig 294. No IBA.

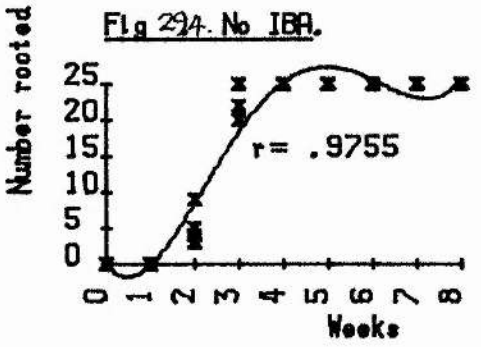


Fig 295. 0.3%IBA + 0.2%BA.

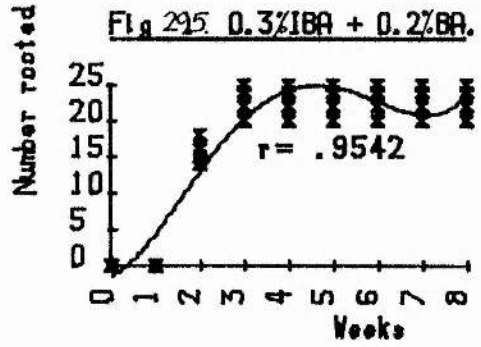


Fig 296. 0.3% IBA + 0.4%BA.

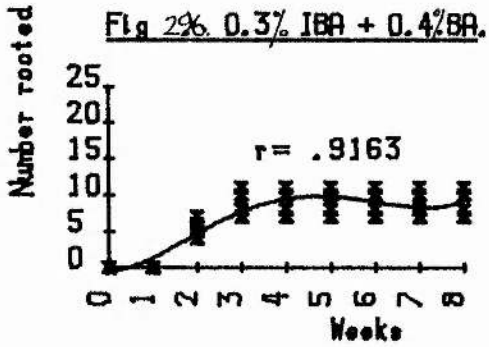


Fig 297. 0.3% IBA.

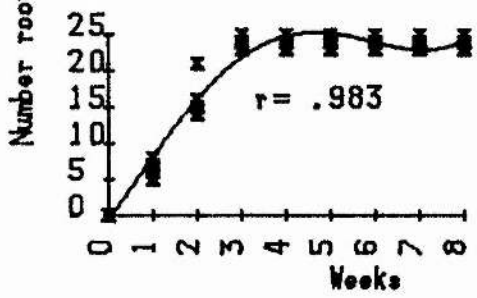


Fig 298. No IBA.

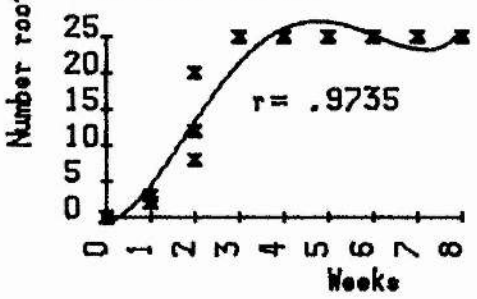


Fig 299. 0.3% IBA

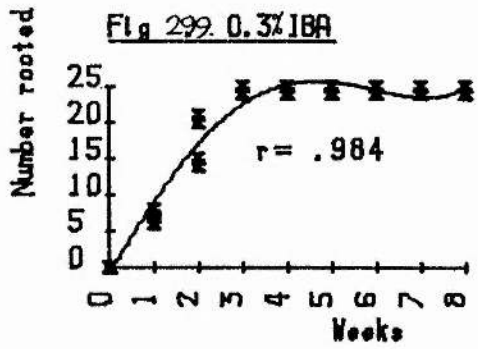


Fig 300. No IBA

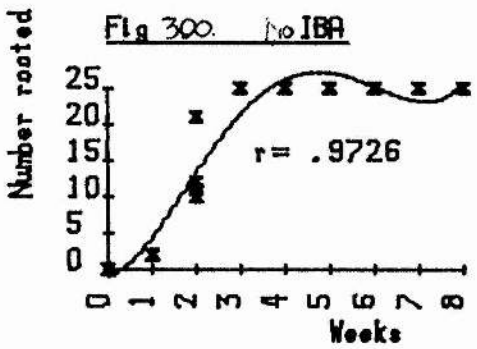


Fig 301. 0.3% IBA.

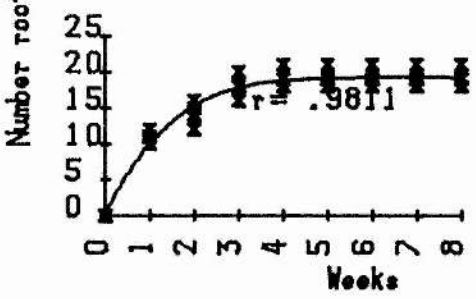


Fig 302. No IBA.

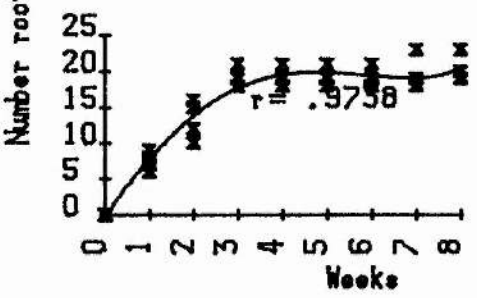


Fig 303. 0.3%IBA

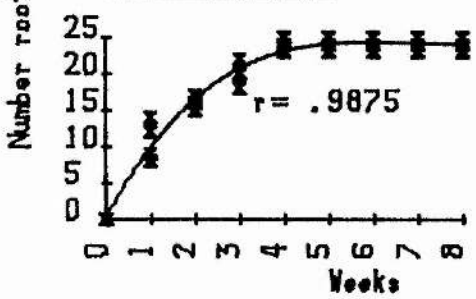


Fig 304. No IBA

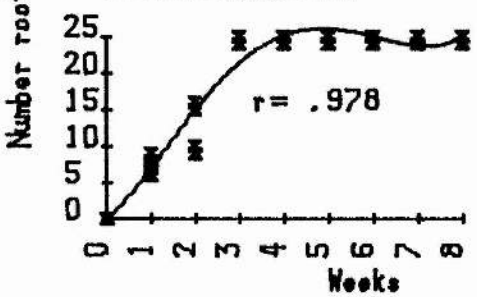


Fig 305. 0.3% IBA.

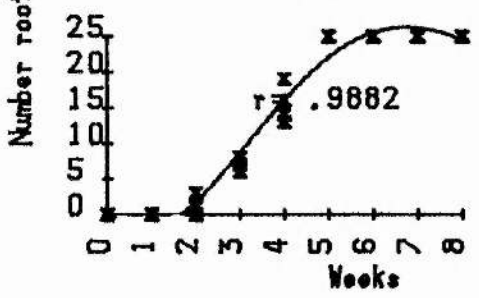


Fig 306. No IBA.

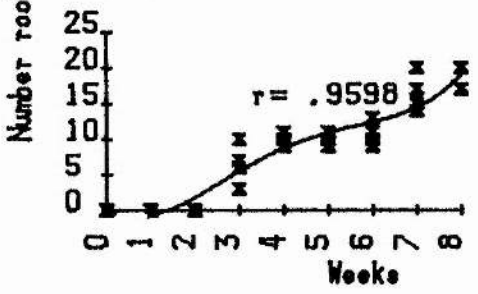


Fig 307. 0.3%IBA + 0.2%BA.

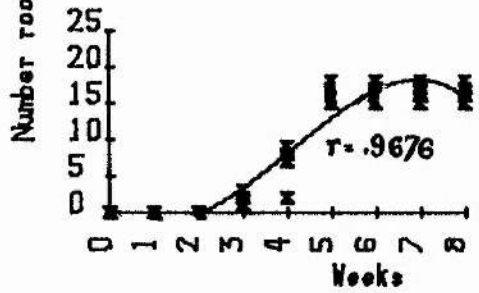


Fig 308. 0.3% IBA + 0.4%BA.

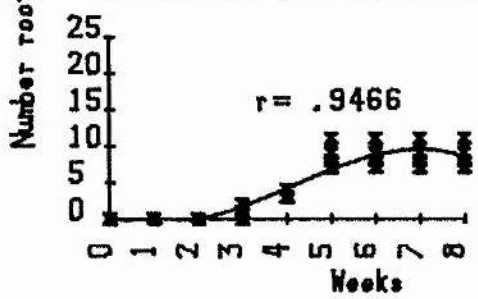


Fig 309. 0.3% IBA.

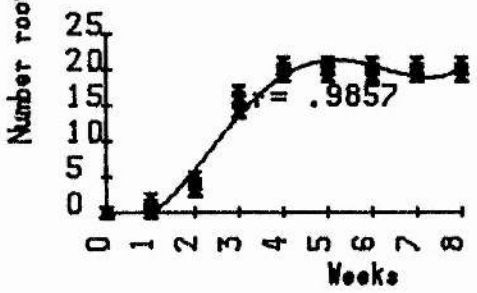


Fig 310. No IBA.

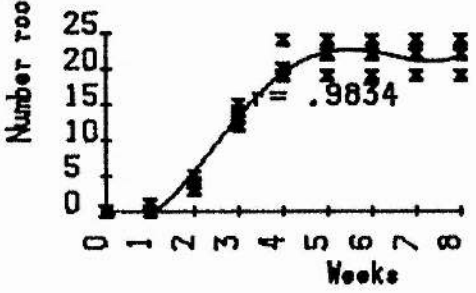


Fig 311. 0.3%IBA

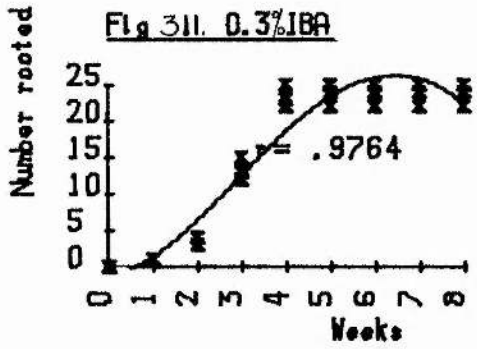


Fig 312. No IBA

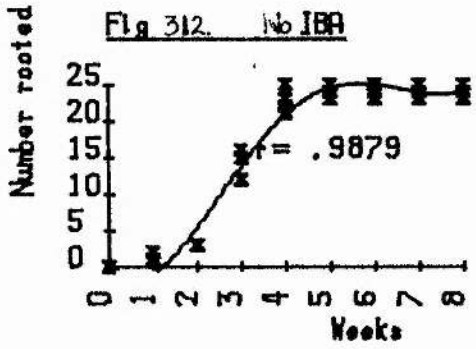


Table 88.

Analysis of variance for data given in Figures 281 to 312.

SOURCE	S.S.	D.F.	M.S.	F	p
Treatment	.02182	7	3.118e-03	164.479	<.001
Species	.02804	3	9.345e-03	493.0375	<.001
Interaction	7.468e-03	21	3.556e-04	18.763	<.001
Error	1.820e-03	96	1.895e-05		
Total	0.5915	127			

Table 89. Mean rate of rooting of macro and mini cuttings of four species : BA and IBA treatment.

Species	Rate of rooting								
	Macro IBA	Macro -IBA	Macro IBA BA.2%	Macro IBA BA.4%	Mini IBA	Mini -IBA	Mini IBA	Mini2 -IBA	Mean
Prunus cerasifera	0.0438	0.0422	0.0420	0.0454	0.0995	0.0765	0.0993	0.0778	0.0658
Spiraea 'Froebelii'	0.0815	0.0563	0.0827	0.0778	0.105	0.0887	0.0931	0.0814	0.0833
Arctostaphylos uva-ursi	0.0621	0.0516	0.0643	0.0625	0.0834	0.0672	0.0857	0.0662	0.0679
Rhododendron 'P.J.M. Victor'	0.0374	0.0331	0.0327	0.0331	0.0519	0.0497	0.0488	0.0487	0.0419
Mean	0.0562	0.0458	0.0554	0.0547	0.0850	0.0705	0.0817	0.0645	0.0647

Rate of rooting given by:- $y/(f*w*7)$ where n=number of weeks, f=final count, w=week of interval

Mini cuttings derived from one in vitro culture period. Mini cuttings 2 derived from BA treated plants.

Macro cuttings - cuttings from untreated whole plants.

L.S.D.=0.0057(body of table), 0.0028(treatment means), 0.0019(species means).

Table 90.

Rate of rooting of macro and mini cuttings (combined data for all species).

Treatment	Rate of rooting
Macro IBA	0.0562d
Macro	0.0458e
Macro IBA BA .2%	0.0554d
Macro IBA BA .4%	0.0547d
Mini IBA	0.0850a
Mini	0.0705c
Mini2 IBA	0.0817b
Mini2	0.0685c

Rate of rooting given by:- $y/(f*w*7)$

where n=number of weeks, f=final count,

w=week of interval

Mini cuttings derived from one in vitro culture period.

Mini cuttings 2 derived from BA treated plants.

Macro cuttings - cuttings from untreated whole plants.

Table 91.

Number of mini cuttings dead at end of experiment 3.

Species	Treatment	Number dead
Prunus cerasifera	+IBA	0, 1, 4, 3
	-IBA	0, 1, 0, 1
Spiraea 'Froebelii'	+IBA	3, 2, 4, 3
	-IBA	1, 1, 0, 2
Arctostaphylos uva-ursi	+IBA	1, 0, 3, 0
	-IBA	0, 0, 0, 0
Rhododendron 'P.J.M. Victor'	+IBA	2, 4, 1, 2
	-IBA	1, 0, 1, 1

Table 92.

Survival of mini cuttings with or without IBA addition.

TREATMENT	DEAD	ALIVE	TOTAL
+IBA	33(21)	367(379)	400
-IBA	9(21)	391(379)	400
TOTAL	42	758	800

Expected values given in parentheses.

$\chi^2 = 14.47$ ($p < .001$)

4.12 ROOT FORMATION IN VITRO

Method

Experiment 1.

Shoot explants of the following species were used :-

Rosaceae:- *Chaenomeles japonica*, *Cotoneaster dammeri*, *Crataegus brachyacantha*, *Crataegus* 'Toba', *Potentilla* 'Coronation Triumph', *Potentilla* 'Sutter's Gold', *Prunus cerasifera*, *Prunus tomentosa*, *Pyracantha coccinea*, *Spiraea* 'Froebelii'.

Ericaceae:- *Arctostaphylos media*, *Arctostaphylos uva-ursi*, *Erica carnea*, *Gaultheria hispidula*, *Kalmia angustifolia*, *Rhododendron arboreum*, *R. chamae-thomsonii*, *R.* 'Chikor', *R.* 'Chinsayii', *R. dauricum*, *R. fastigiatum*, *R. forrestii*, *R. keiskei*, *R. leucaspis*, *R. lutescens*, *R.* 'P.J.M. Victor', *R. racemosum*, *R.* 'Vuyk's rosy red', *R. williamsianum*, *Vaccinium vitis-idaea*.

IBA at 0, 0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg l^{-1} was incorporated in the nutrient medium.

Cultures of Rosaceae were incubated a) in light (16 hour photoperiod), b) in darkness for one week followed by light incubation (16 hour photoperiod) or c) in continuous darkness. Cultures of Ericaceous species were incubated in light treatment (b). Root number was recorded at the end of a four week incubation period.

Cultures incubated in continuous darkness were incubated for a further two weeks in light (16 hour photoperiod) to determine whether darkness promotes root initiation but inhibits root growth i.e. visible root formation. Root number was recorded at the end of this period.

Experiment 2. Shoot explants of Prunus cerasifera and Spiraea 'Froebelii' were used. IBA was incorporated in the medium as detailed in Experiment 1. Activated charcoal at 0, 1, 2, 3, 4 or 5 g l^{-1} was added to darken the medium although the use of charcoal may have other additional effects such as absorption of grwoth regulators or phenolic coompounds. Cultures were incubated in light (16 hour photoperiod).

Root number was recorded at the end of a two week

incubation period.

Results

Experiment 1.

The IBA concentration which stimulated the formation of the greatest number of roots varied with species from 1.0 to 10.0 mg l (Tables 93 to 95 for Rosaceae; Table 96 for Ericaceae).

An analysis of variance showed a significant effect on root number due to IBA concentration ($p<.001$), a significant effect due to species ($p<.001$), a significant effect due to light treatment ($p<.001$) and a significant interaction between these ($p<.001$) in Rosaceae (Table 97). The order of effectiveness of light treatment was (1) light / dark, (2) dark, (3) light (Table 98). Order of effectiveness of IBA concentration in shoot formation was (1) 1.0, (2) 0.5, 2.5, (3) 5.0, (4) 10.0, (5) 0, 20.0 mg l^{-1} (Table 99). Species varied in capacity for root formation under the conditions of the experiment (Table 100).

An analysis of variance for Ericaceae showed a significant effect on root formation due to IBA

concentration ($p<.001$), species ($p<.001$) and a significant interaction between these ($p<.001$) (Table 101). Order of effectiveness of IBA concentration on rooting is given in Table 102 - most roots were formed at 1.0 mg l^{-1} IBA and least at 0 and 20.0 mg l^{-1}. A range of rooting potential among species was demonstrated (Table 103). Most roots were formed in Arctostaphylos species while no roots formed in Rhododendron arboreum, R. forrestii or R. williamsianum.

Explants maintained in continuous darkness for 4 weeks developed good root systems but the shoot did not grow and turned yellow.

Plate 14 shows root development in vitro. Roots were shorter at high than at low IBA concentrations. Roots of Cotoneaster were pink and did not grow into the medium (Plate 15). Roots of other species were white initially but darkened within a week of growth into the medium. Root hairs were present in all species although their frequency was observed to be less than in roots formed in soil.

Root number was not increased in any species by light incubation following a four week dark incubation period.

Experiment 2.

Root number was decreased when activated charcoal was incorporated in the medium in Spiraea (Tables 104 and 106) but was slightly increased in Prunus (Table 107). An analysis of variance for Spiraea showed a significant effect on root formation due to charcoal concentration ($p<.001$), IBA concentration ($p<.001$) and a significant interaction ($p<.001$) between these (Table 105). An analysis of variance for Prunus showed a significant effect of charcoal concentration ($p<.05$) and IBA concentration ($p<.05$) on root initiation, but no interaction between charcoal and IBA (Table 108). In Prunus, most roots were formed at 2 g l^{-1} charcoal (Table 109).

Table 93. Mean root number after 4weeks incubation on medium containing IBA : Rosaceae 16 hour daylength

Species	IBA concentration (mg l^{-1})						
	0	0.5	1.0	2.5	5.0	10.0	20.0
Chaenomeles japonica	0	0	0	0	0.75	2.0	3.0
Cotoneaster dammeri	0	0	0.5	1.75	1.25	0	0
Crataegus brachyacantha	0	0	1.25	1.5	4.25	4.75	0
Crataegus 'Toba'	0	0	0	0	1.5	2.0	0.75
Potentilla 'Coronation Triumph'	1.0	6.75	4.5	3.5	0	0	0
Potentilla 'Sutter's Gold'	2.05	8.75	3.75	2.25	2.25	0	0
Prunus cerasifera	0	0	0	1.0	0.5	0	0
Prunus tomentosa	0	0	0	0	0	0	0
Pyracantha coccinea	0	0	0	0	0	0	0
Spiraea 'Froebelii'	0	14.5	20.25	5.75	2.5	0.5	0

Table 94. Mean root number after 4 weeks incubation on IBA medium : Rosaceae Dark 1 week followed by 16 hour daylength.

Species	IBA concentration (mg l^{-1})						
	0	0.5	1.0	2.5	5.0	10.0	20.0
Chaenomeles japonica	0	0	0	0.25	1.5	1.25	0
Cotoneaster dammeri	0	0	0.25	2.75	3.25	0	0
Crataegus brachyacantha	0	0.5	1.0	5.5	4.5	1.0	0
Crataegus 'Toba'	0	0	0	1.0	3.75	5.75	2.0
Potentilla 'Coronation Triumph'	1.25	5.75	6.0	4.75	1.25	0	0
Potentilla 'Sutter's Gold'	1.75	7.0	9.25	3.0	1.25	0	0
Prunus cerasifera	0	3.75	4.75	9.25	4.0	0	0
Prunus tomentosa	0	0	0	2.25	1.0	0	0
Pyracantha coccinea	0	0	0	0	2.25	2.75	0.75
Spiraea 'Froebelii'	0.25	13.5	20.5	7.5	2.0	0.25	0

Table 95. Mean root number after 4 weeks incubation on IBA medium : Rosaceae : Continuous darkness.

Species	IBA concentration (mg l^{-1})						
	0	0.5	1.0	2.5	5.0	10.0	20.0
Chaenomeles japonica	0	0	0	0.25	3.75	0	0
Cotoneaster dammeri	0	0	0.5	2.0	3.75	0	0
Crataegus brachyacantha	0	0.25	0.75	4.25	4.5	0.75	0
Crataegus 'Toba'	0	0	0	0.75	3.0	6.05	2.25
Potentilla 'Coronation Triumph'	0.25	1.25	4.75	5.5	0.75	0.25	0
Potentilla 'Sutter's Gold'	0.75	1.75	6.75	3.0	1.25	0	0
Prunus cerasifera	0	3.5	7.0	8.75	4.0	0	0
Prunus tomentosa	0	0	0	2.5	0.75	0	0
Pyracantha coccinea	0	0	0	0	8.0	2.5	0.25
Spiraea 'Froebelii'	0.75	12.75	19.25	3.25	0	0	0

Table 96.Mean root number after 4 weeks incubation of IBA medium :

Ericaceae : Dark for one week followed by a 16 hour daylength.

Species	IBA concentration (mg l^{-1})						
	0	0.5	1.0	2.5	5.0	10.0	20.0
Arctostaphylos media	0	0.75	10.5	5.0	2.5	0.75	0
Arctostaphylos uva-ursi	0	0	1.5	4.0	12.75	5.5	0.25
Erica carnea	0	2.25	15.5	4.225	0	0	0
Gaultheria hispidula	0	0	0	0.75	1.50	0	0
Kalmia angustifolia	0	0	2.0	3.75	0	0	0
Rhododendron arboreum	0	0	0	0	0	0	0
Rhododendron chamae-thomsonii	0	0	0	2.5	0.5	0	0
Rhododendron 'Chikor'	0	2.0	10.25	4.5	0.25	0	0

Table 96 continued.

Rhododendron 'chinsayii'	0	0	0	0	3.5	16.0	0.75
Rhododendron dauricum	0	0	2.5	5.25	6.75	0.75	0
Rhododendron fastigiatum	0	0.25	3.75	1.25	0	0	0
Rhododendron forrestii	0	0	0	0	0	0	0
Rhododendron keiskei	0	0	0	1.0	0.75	0	0
Rhododendron leucaspis	0	0	0	0	1.25	0	0
Rhododendron lutescens	0	0	0	0	2.25	16.75	0.5
Rhododendron 'P.J.M.Victor'	0	0	0	0.75	0.5	2.5	1.5
Rhododendron racemosum	0	0	2.0	0.5	0	0	0
Rhododendron 'Vuyk's'	0	0	0.75	0	0	0	0
Rhododendron williamsianum	0	0	0	0	0	0	0
Vaccinium vitis-idaea	0	2.5	2.0	0.5	0	0	0

Table 97.

Analysis of variance for data presented in Tables 93 to 95.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA	1426.581	6	237.763	264.532	<.001
Species	2194.963	9	243.885	271.342	<.001
Light	67.674	2	33.837	37.646	<.001
IBA * species	5417.229	54	100.319	111.613	<.001
IBA * light	127.176	12	10.598	11.791	<.001
Species * light	224.183	18	12.455	13.857	<.001
IBA * species * light	574.800	108	5.322	1.310	<.05
Error	566.250	630	0.899		
Total	10598.856	839			

Table 98.

Mean number of roots formed per plant after 16 hour photoperiods, 1 week darkness followed by 16 hour photoperiods or continuous darkness : Rosaceae.

Light treatment	Mean root number
16 hour photoperiod	1.55c
1 week dark,	2.24a
+ 16 hour photoperiod	
Continuous darkness	1.85b

($p<.05$)

Table 99.

Mean number of roots formed per plant as a function of IBA concentration : Rosaceae.

mg l^{-1} IBA	Mean root number
0	0.277e
0.5	2.72b
1.0	3.93a
2.5	2.91b
5.0	2.04c
10.0	0.99d
20.0	0.30e

($p<.05$)

Means followed by different letters are significantly different.

Table 100.

Mean root number : Rosaceae.

Species	Number of roots
Chaenomeles japonica	0.51fg
Cotoneaster dammeri	0.67f
Crataegus brachyacantha	1.85d
Crataegus 'Toba'	1.32e
Potentilla 'Coronation Triumph'	2.73b
Potentilla 'Sutter's Gold'	2.68b
Prunus cerasifera	2.29c
Prunus tomentosa	0.31g
Pyracantha coccinea	0.50fg
Spiraea 'Froebelii'	5.95a

Means followed by a different letter are significantly different ($p<.05$).

Table 101.

Analysis of variance for data given in Table 96.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	46.398	6	7.733	125.775	<.001
Species	57.513	19	3.027	49.233	<.001
Interaction	177.765	114	1.559	25.362	<.001
Error	25.823	420	0.0615		
Total	307.499	559			

A loge (y+1) transformation was used.

Table 102.

Mean number of roots formed per plant as a function of IBA concentration : Ericaceae.

mg l^{-1} IBA	Mean root number
0	0e
0.5	0.388d
1.0	2.54a
2.5	1.83ab
5.0	1.90b
10.0	1.99c
20.0	0.15e

Means followed by different lettera are significantly different

A loge (y+1) transformation was used.

Table 103.

Mean root number : Ericaceae.

Species	Root number
Arctostaphylos uva-ursi	3.46
Arctostaphylos media	3.07
Erica carnea	3.21
Rhododendron dauricum	2.18
Rhododendron 'chikor'	2.43
Rhododendron 'chinsayii'	2.53
Rhododendron lutescens	2.89
Rhododendron 'P.J.M. Victor'	1.39
Vaccinium vitis-idaea	0.71
Kalmia angustifolia	0.82
Rhododendron fastigiatum	0.75
Rhododendron chamae-thomsonii	0.43
Rhododendron racemosum	0.36
Gaultheria hispidula	0.32
Rhododendron keiskei	0.25
Rhododendron leucaspis	0.18
Rhododendron 'Vuyk's'	0.11
Rhododendron arboreum	0
Rhododendron forrestii	0
Rhododendron williamsianum	0

Species not bounded by a common line are significantly different

($p<.05$ using $\log_e (y+1)$ transformation).

Table 104. Mean root number after a 4 week incubation period on medium containing charcoal : Spiraea 'Froebelii'.

IBA mg l^{-1}	Charcoal g l^{-1}						
	0	1	2	3	4	5	Mean
0	0	0	0	1.0	0.25	0	0.21
0.1	3.5	1.5	1.75	2.5	0.25	0	1.58
0.5	14.5	2.75	3.0	6.25	4.0	1.0	5.25
1.0	20.25	2.5	5.25	10.5	4.75	2.75	7.67
2.5	7.25	4.0	4.0	6.5	3.0	1.5	4.4
5.0	1.75	0.5	0.75	1.5	0	0	0.75
10.0	0.5	0.25	0	0	0	0	0.13
20.0	0	0	0	0	0	0	0
Mean	5.97	1.44	1.84	3.53	1.53	0.66	2.49

L.S.D=1.37 (body of table), 0.48 (charcoal means)

Table 105. Analysis of variance for data presented in Table 104.

SOURCE	S.S.	D.F.	M.S.	F	p
Charcoal	20.064	5	4.013	79.020	<.001
IBA conc.	102.569	7	14.653	288.542	<.001
Interaction	15.333	35	0.438	8.627	<.001
Error	7.313	144	0.508		
Total	145.279	191			

A loge (y+1) transformation was used.

Table 106. Change in mean root number with concentration of charcoal in medium after a 4 week incubation period : Spiraea 'Froebelii'.

Charcoal g l^{-1}	Mean root number
0	5.97a
1	1.44c
2	1.84c
3	3.53b
4	1.53c
5	0.66d

Table 107. Mean root number after 4 weeks incubation on medium containing charcoal : Prunus cerasifera.

IBA mg l^{-1}	Charcoal g l^{-1}						
	0	1	2	3	4	5	Mean
1.0	0	0	0.75	0.25	0	0	0.17
2.5	1.0	0.5	1.5	0	0	0	0.5
5.0	0.25	0.25	0	0	0	0	0.08
Mean	0.42	0.25	0.75	0.08	0	0	

L.S.D. = 0.86 (body of table), 0.50 (charcoal means) and 0.35 for IBA means.

Table 106. Change in mean root number with concentration of charcoal in medium after a 4 week incubation period : Spiraea 'Froebelii'.

Charcoal g l^{-1}			Mean root number		
0	5.97a	1	1.44c	2	1.84c
3	3.53b	4	1.53c	5	0.66d

Table 107. Mean root number after 4 weeks incubation on medium containing charcoal : Prunus ceras ifera.

IBA mg l	Charcoal g l					
	0	1	2	3	4	5Mean
1.0	0	0	0.75	0.25	0	00.17
2.5	1.0	0.5	1.5	0	0	00.5
5.0	0.25	0.25	0	0	0	00.08
Mean	0.42	0.25	0.75	0.08	0	0

L.S.D. = 0.86 (body of table), 0.50 (charcoal means) and 0.35 for IBA means.

Table 108.

Analysis of variance for data given in Table 107.

SOURCE	S.S.	D.F.	M.S.	F	P
Charcoal conc.	5.167	5	1.033	2.937	<.05
IBA conc	2.333	2	1.167	3.316	<.05
Interaction	5.000	10	0.500	1.421	N.S.
Error	19.000	54	0.352		
Total	31.500	71			

No transformation was used. When a loge (y+1) transformation was used then IBA effect was not significant.

Table 109.

Mean number of roots formed per plant as a function of charcoal concentration in medium : Prunus cerasifera.

g l^{-1} charcoal	Mean root number
0	0.417b
1	0.250bc
2	0.750a
3	0.083cd
4	0d
5	0d

Means followed by different letters are significantly different ($p<.05$).

Plate 14.

Root formation in Prunus cerasifera.

Plate 15.

Root formation in Cotoneaster dammeri - roots growing in air space.

4.13 DYNAMICS OF IN VITRO ROOT FORMATION

Method

Experiment 1.

The following species were used in the experiment :- Rosaceae : *Crataegus brachyacantha*, *Prunus cerasifera*, *Spiraea* 'Froebelii'; Ericaceae : *Arctostaphylos uva-ursi*, *Rhododendron* 'chikor', *Rhododendron* 'PJM Victor'. IBA was incorporated in the nutrient medium at the concentrations which induced maximal root formation (Section 4.12).

Root number was recorded at the end of 1, 2, 3, 4, 5, 6, 7 and 8 weeks incubation in light treatment (b) (see Section 4.12) - one week in darkness followed by light incubation (16 hour photoperiod).

Experiment 2.

Species tested in this experiment were the same as for Experiment 1 above. IBA was supplied in the nutrient medium at the concentrations which induced maximal root formation (Section 4.12) for 1, 3, 5, 7, 14, 21 or 28 days. Plant material was then placed on nutrient medium without growth regulators for the

remainder of the 4 week culture period. Root number was recorded at the end of this period. Incubation was as specified for Experiment 1 above.

Results

Experiment 1.

Fitted curves for rate of root formation were plotted ($p<.001$) (Figures 313 to 318). An analysis of variance showed a significant effect on root formation due to number of weeks in culture ($p<.001$), species ($p<.001$) and a significant interaction between time for rooting and species ($p<.01$) (Table 110). Most roots formed in the second week of culture (Table 111).

Experiment 2.

Fitted curves were plotted for number of roots formed against exposure period to exogenous IBA ($p<.001$ in all cases for fit of curve) (Figures 319 to 324). An analysis of variance showed a significant effect on root formation due to exposur period to IBA ($p<.001$), a significant effect due to species ($p<.001$) and a significant interaction between these ($p<.001$) (Table

112). Root number increased with increasing exposure period to IBA (Table 113).

Table 110.

Analysis of variance for data given in Figures 313 to 318.

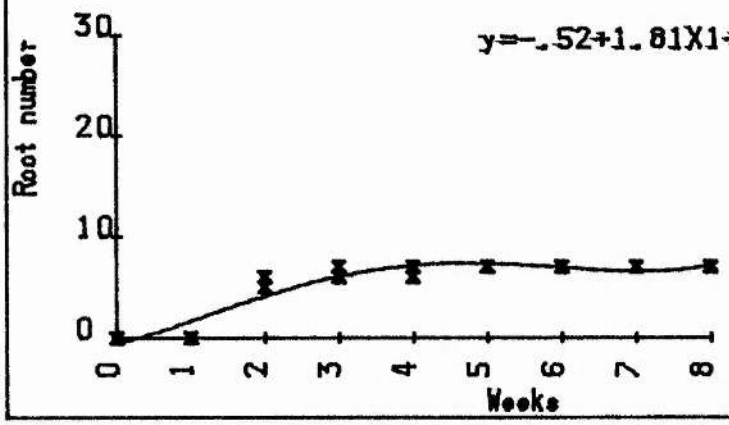
SOURCE	S.S.	D.F.	M.S.	F	p
Weeks	195.566	8	24.446	410.929	<.001
Species	24.511	5	4.902	82.406	<.001
Interaction	8.913	40	0.222	3.746	<.001
Error	9.637	162	0.059		
Total	238.628	215			

A loge (y+1) transformation was used.

Figures 313 to 318.

Rate of root formation on medium containing IBA.

Fig 313. Crataegus brachyacantha.



y=-.52+1.81X1+.62X2-.21X3+.01X4

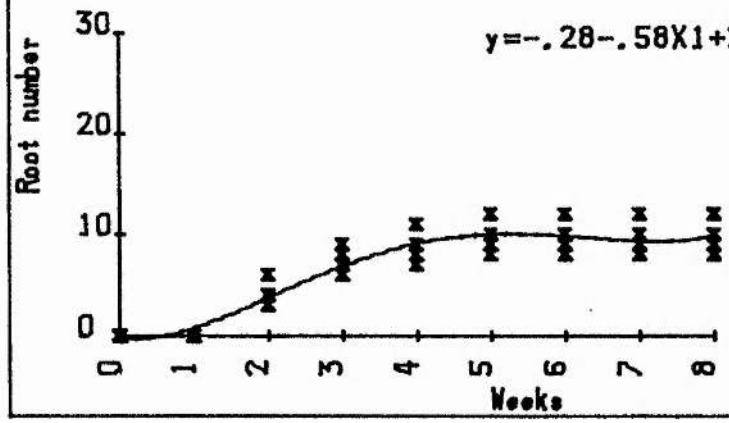
R2= .8961

r= .9466

(p=<.001)

S.E.= .978

Fig 314. Prunus cerasifera.



y=-.28-.58X1+2.07X2-.45X3+.02X4

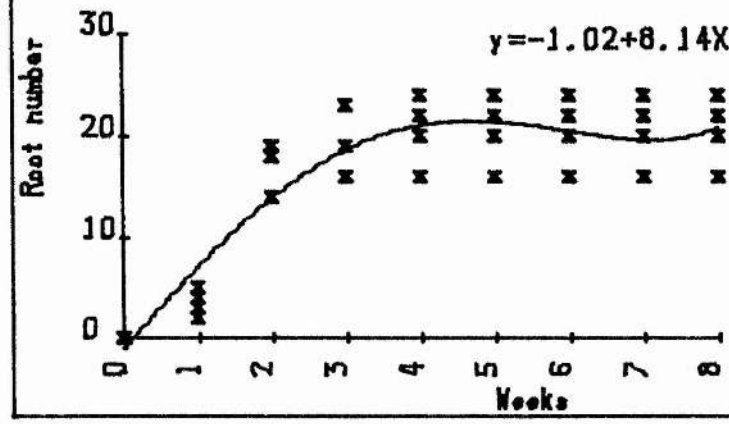
R2= .8995

r= .9484

(p=<.001)

S.E.= 1.399

Fig 315. Spiraea 'Froebellii'.



y=-1.02+8.14X1+.16X2-.31X3+.02X4

R2= .8511

r= .9225

(p=<.001)

S.E.= 3.334

Fig 316. Arctostaphylos uva-ursi.

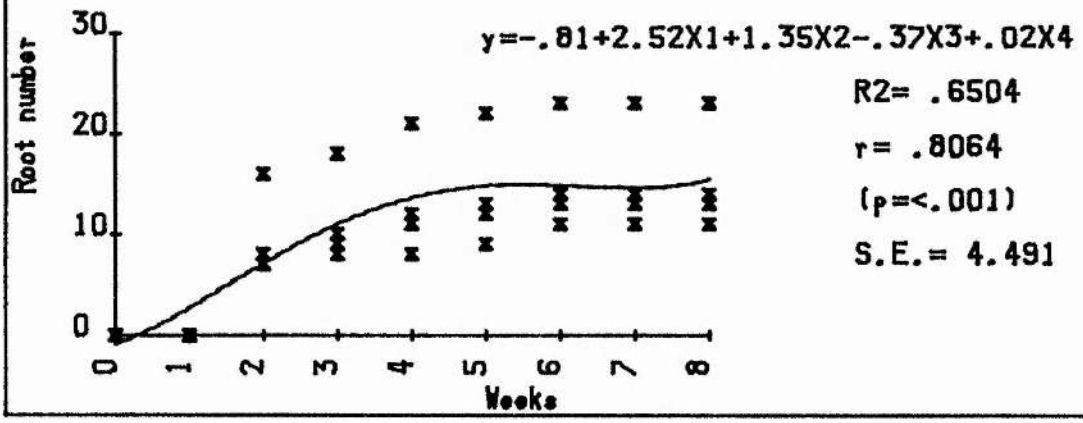


Fig 317. Rhododendron chlkor.

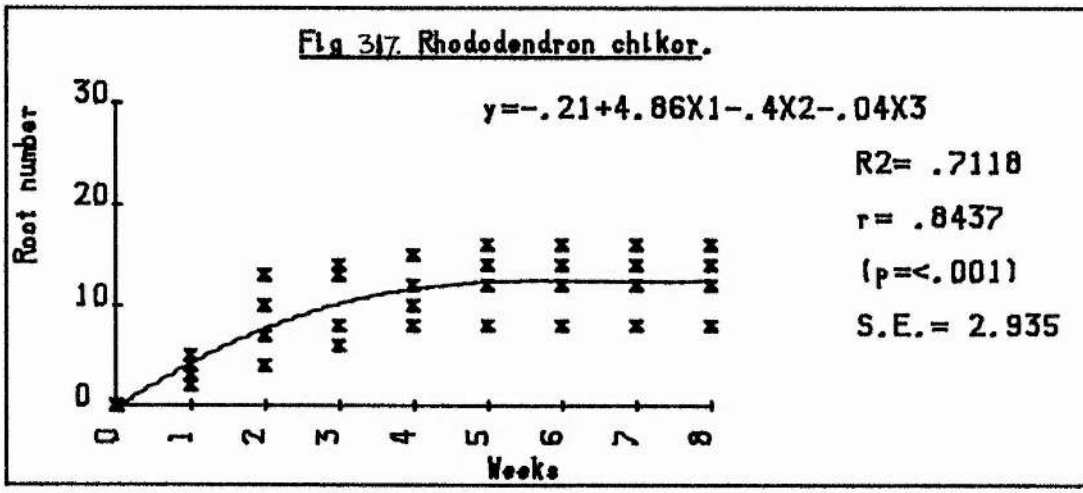
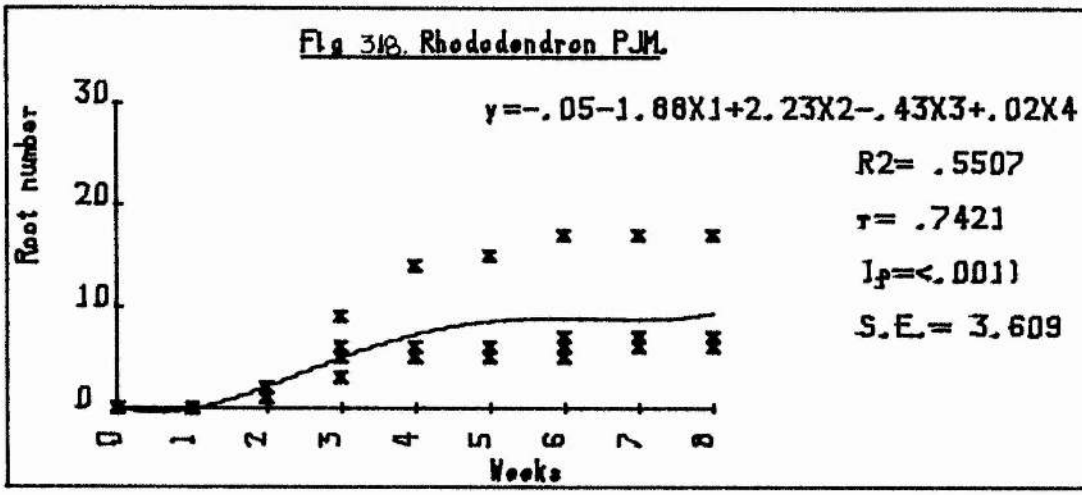


Fig 318. Rhododendron PJM.



Figures 319 to 324.

Mean number of roots formed in four weeks after varying incubation periods on medium containing IBA.

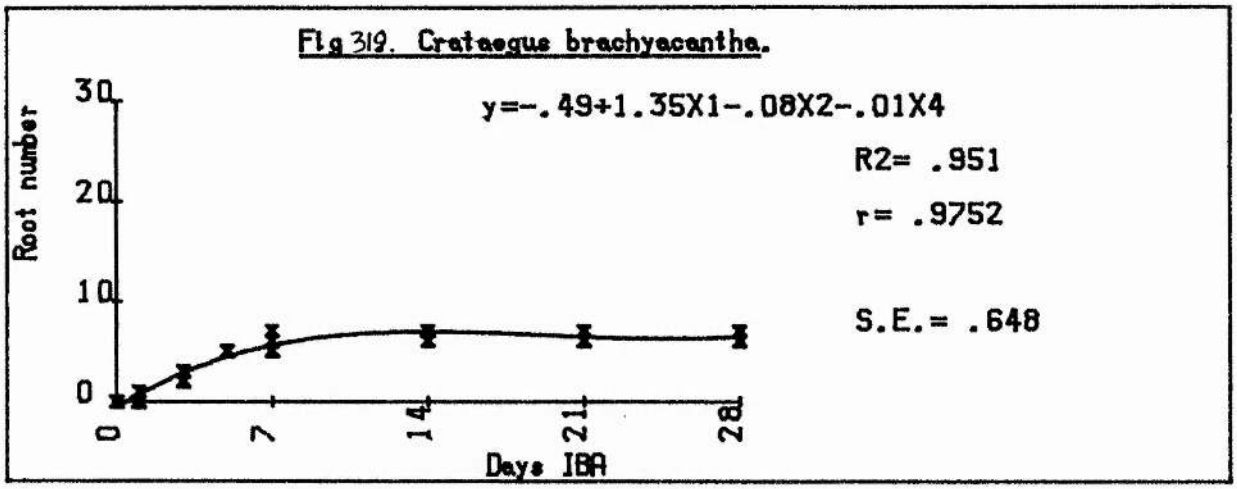


Fig 319. Crataegus brachyacantha.
y=-.49+1.35X1-.08X2-.01X4
R2= .951
r= .9752
S.E.= .648
Root number
30
20
10
0
0
7
14
21
28
Days IBA

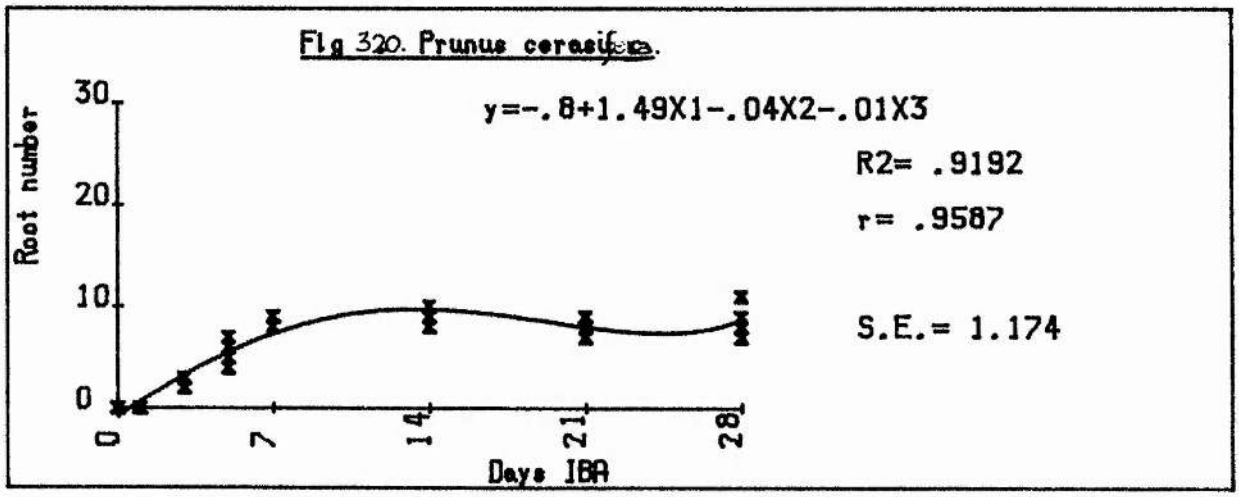


Fig 320. Prunus cerasifera.
y=-.8+1.49X1-.04X2-.01X3
R2= .9192
r= .9587
S.E.= 1.174
Root number
30
20
10
0
0
7
14
21
28
Days IBA

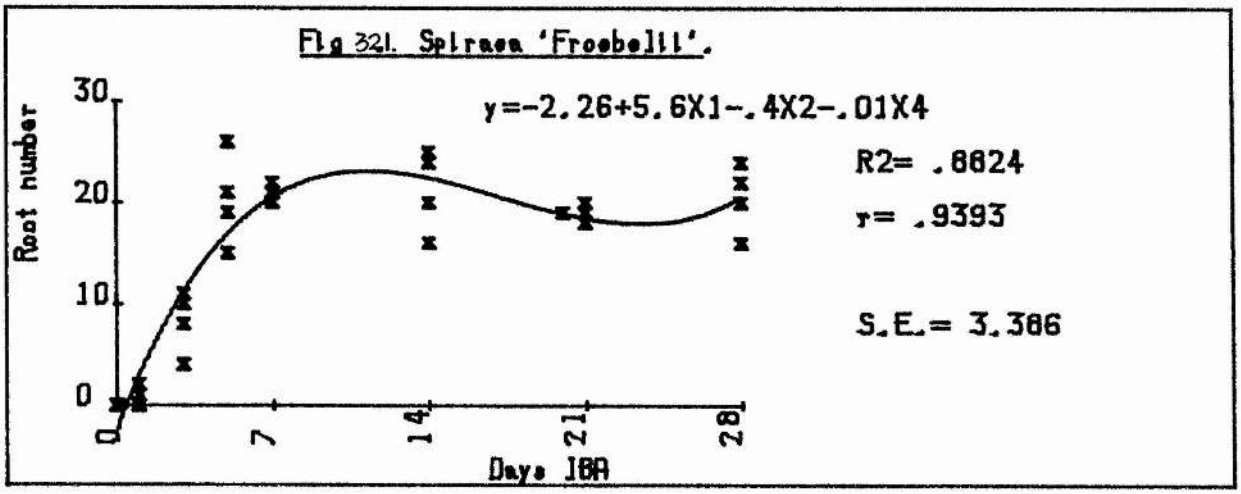


Fig 321. Spiraea 'Froebelii'.
y=-2.26+5.6X1-.4X2-.01X4
R2= .8624
r= .9393
S.E.= 3.386
Root number
30
20
10
0
0
7
14
21
28
Days IBA

Fig 322. Arctostaphylos uva-ursi.

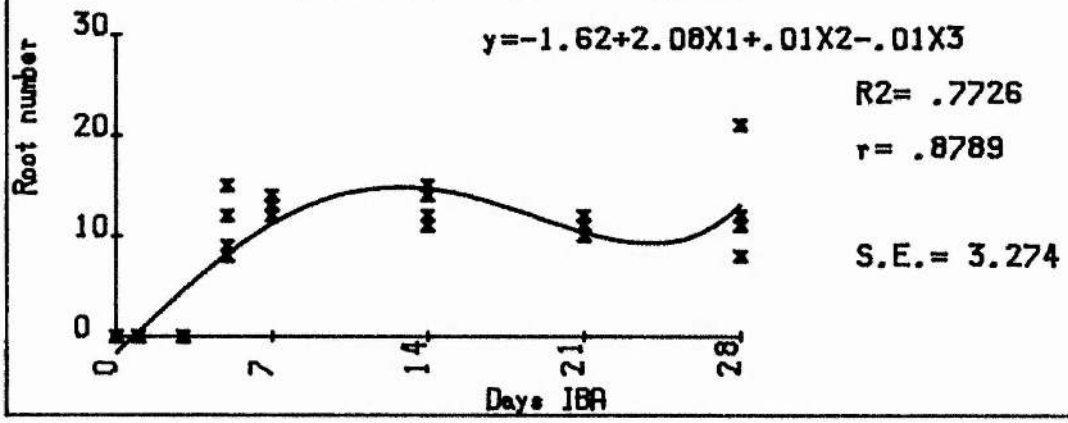


Fig 323. Rhododendron chikor.

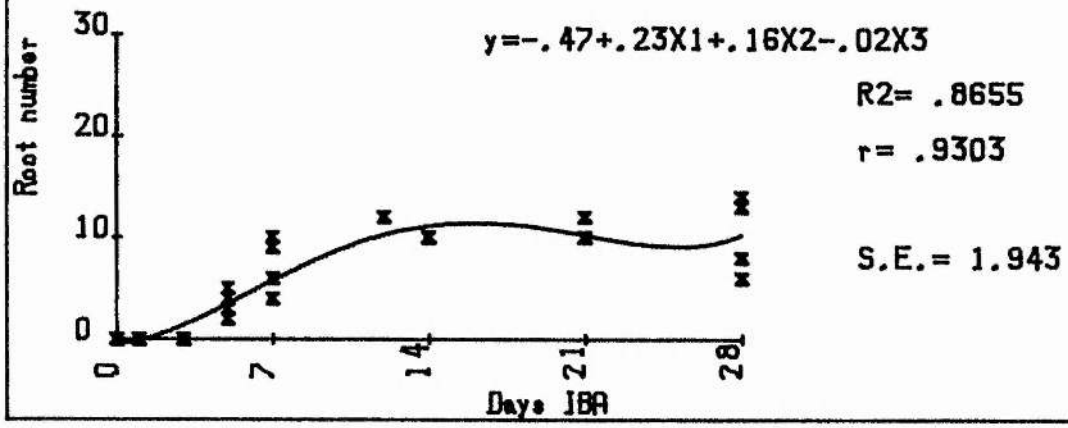


Fig. 324 Rhododendron PJM.

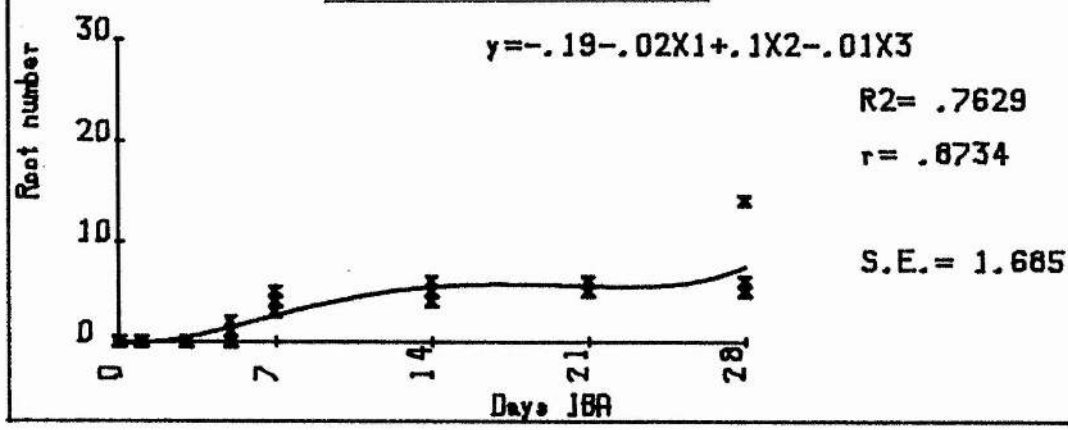


Table 111.

Change in mean number of roots formed per plant with time : All species combined.

Weeks	Mean root number
0	0d
1	0.58d
2	7.08c
3	9.83b
4	11.08ab
5	11.75a
6	12.29a
7	12.38a
8	12.38a

Means followed by different letters are significantly different ($p<.05$)(based on loge(y+1) transformation).

Table 112.

Analysis of variance for data given in Figures 319 to 324.

SOURCE	S.S.	D.F.	M.S.	F	p
Days	181.039	7	25.863	374.251	<.001
Species	23.815	5	4.763	68.922	<.001
Interaction	22.308	35	0.637	9.223	<.001
Error	9.951	144	0.069		
Total	237.113	191			

A loge (y+1) transformation was used.

Table 113.

Change in mean number of roots formed per plant with time of exposure to IBA : All species combined.

Days	Mean root number
0	0f
1	0.17e
3	2.21d
5	7.25c
7	9.96b
14	10.83a
21	9.54b
28	11.08a

Means followed by different lettera are significantly different ($p<.05$)(based on loge(y+1) transformation).

4.14 DISCUSSION

Root formation in stem cuttings in greenhouse conditions

Considerable differences in rooting were observed between species both in the presence and absence of auxin. Differences have previously been noted, for example, Thimann and Delisle (1942) recorded that some conifers readily formed roots on stem cuttings whereas others did not; in the family Ericaceae, Ledum palustre does not form adventitious roots readily, whereas Calluna vulgaris does so readily (Komissarov, 1969); and in the family Rosaceae, 'Bartlett' pears are difficult-to-root, while 'East Malling XIII' apples are easy-to-root from stem cuttings (Beakbane, 1961).

It has been postulated by several workers that differences between woody species in potential for adventitious root formation are due to anatomical differences between species (Ciampi and Gellini, 1958, Beakbane, 1961, 1969). Specifically, Beakbane proposed that the anatomical structure of the primary phloem is critical for adventitious root formation. For example, cuttings of 'Conference' and 'Bartlett' pear are

difficult to root and they have an almost continuous cylinder of mature thick-walled fibre cells encircling the secondary phloem, whereas, in easily rooted cultivars such as 'EM V', 'EM XIII' apples, this sclerenchyma ring is not continuous and would permit the emergence of roots formed inside the ring.

However, Sachs et al. (1964) showed that there was no similar relationship for olive, cherry and pear cuttings between continuity of a sclerenchyma ring and rooting potential; and Hartmann et al. (1963) showed that 'Bartlett' pear cuttings which have an almost continuous sclerenchyma ring, can be rooted in high percentages if suitable environmental conditions are provided.

Some species have preformed root primordia, for example, willow and poplar (Hartmann, 1969), in their stems. However, an examination of several of the test species did not reveal any such primordia. Therefore, based on the above evidence, it is deduced that stem anatomy is unlikely to account for differences in the observed rooting potential.

An IBA quick dip increased the percentage of cuttings forming roots in nearly half of the species tested. However, rooting was decreased in relation to

the control in some species. These results show that rooting can be both enhanced and decreased by the application of auxin. Only one concentration of IBA was applied in this experiment and therefore, root initiation could be governed by auxin concentration, different species having different requirements in this respect.

The second experiment tested the effect of auxin concentration on root initiation. Species differences in response were apparent - whereas auxin at all concentrations tested decreased root initiation in Arctostaphylos, an increase was recorded in the other three species at IBA concentrations of 0.3% or less. High IBA levels decreased rooting in all species. It has been proposed (Donnelly, 1971) that rooting response to applied auxin is in direct opposition to the apparent endogenous auxin content. If this is so, and if auxin concentration controls rooting, then either Arctostaphylos has a higher endogenous auxin content than the other species or different species require different total auxin concentrations to stimulate maximal root initiation.

Mini cuttings initiated roots more quickly than macro cuttings. As mini cuttings from in vitro

and whole plant sources both demonstrated faster root initiation, environmental and nutritional factors can be discounted as causal agents of this phenomenon.

Preconditioning for faster root initiation may therefore be due to cytokinin (either directly or indirectly). This was investigated further by applying cytokinin and auxin simultaneously to macro cuttings. However, in this case, no increase in speed of root initiation was observed thus indicating that sequential action of cytokinin followed by auxin may be important in determining rate of root formation. Cytokinin pretreatment could cause the formation of non-determined organ initials but a microscopic examination did not reveal any such groups of cells; or when auxin alone is applied, an additional intermediate process may be occurring.

When cytokinin and auxin were applied simultaneously to macro-cuttings, cytokinin was applied to the base of the cutting whereas cytokinin pretreatment of mini-cuttings was to the entire length of the stem (if uptake is via the entire surface area in contact with the medium - discussed in Chapters 3 and 5). However, as BA transport is principally acropetal in shoots (references cited in Section 3.1),

it is unlikely that this possible difference would affect the outcome of the experiment.

Intact plants failed to initiate adventitious roots after auxin application. This demonstrates that excision or mechanical wounding, or nutrient or carbohydrate status may affect adventitious root formation in the species tested.

Root formation in vitro

Similar variation between species to that observed in greenhouse experiments was also recorded in vitro. Results from experiments in which IBA concentration was varied, again demonstrated that, if auxin concentration is the determining factor in root initiation, then either species must differ in their endogenous auxin content or in the total auxin concentration required for root determination.

Light and rooting in vitro

Some species failed to regenerate roots when incubated under supplemental lighted conditions, but rooted successfully when placed in continuous darkness for one week (prior to incubation in light). Similar findings have been reported by other workers. For example, Pierik (1969) showed that light inhibited and

continuous darkness promoted root initiation in two Rhododendron cultivars grown *in vitro*, and light has also been shown to inhibit adventitious root initiation in other species (Galston, 1948; Leroux, 1967; Nanda et al., 1968; Pierik, 1970) . On the other hand, Spanjersberg and Gautheret (1963) showed that adventitious root formation in isolated tuber tissues of *Helianthus* was strongly promoted by light as was root initiation in isolated tissue of other species (Gautheret, 1969; Letouze and Beauchesne, 1969; Lovell and Moore, 1969; Rucker and Paupardin, 1969; Weis and Jaffe, 1969).

In the species tested here, darkness for a period longer than eight hours was essential to the root initiation process. Additionally, those species which did root under lighted (16 hour photoperiod) conditions, initiated more roots in darkness. The work of Olieman van der Meer et al. (1970) supports this - they found that increasing exposure time to light decreased the number of roots formed in *Rhododendron*. However, in my experiments, the number of roots formed was, in all species, less when incubated in continuous darkness for four weeks than when incubated in continuous darkness for only one week. It is possible,

therefore, that darkness may promote root initiation (not visible to the naked eye) but inhibit root growth . However, cultures incubated in darkness for four weeks and then incubated in light failed to form any additional roots and so this is unlikely.

Shoots did not grow, and senesced in continuous darkness and, therefore, shoot condition may limit root initiation in prolonged darkness. Altman (1972) demonstrated the importance of leaves in rooting and this suggests that carbohydrate supply is important in determining root initiation. This is discussed further in Chapter VI. The occurrence of senescence suggests that ethene synthesis may be taking place in darkness. Ethene synthesis and the role of ethene in root initiation is investigated in Chapter VIII. Another possibility is that carbon dioxide content of the air in the culture tube might increase in darkness (due to lack of photosynthesis). This could be readily absorbed into the medium and the plant tissue resulting in carbonic acid formation and thus a decrease in pH and an increase in hydrogen ion concentration. A low pH favours root formation (see Chapter VI).

The point at which darkness may affect root initiation is the actual site of root initiation rather

than the whole cutting. This is deduced from the fact that species which failed to initiate roots in light in vitro, rooted successfully in greenhouse conditions with similar lighting conditions provided to the top of the cutting only (the base where roots formed being in darkness in the rooting medium). This has also been demonstrated in other species, for example, Mevius (1931) showed that rooting of Tradescantia cuttings was inhibited when the bases were exposed to light.

Several theories to explain the failure of light-grown explants to root have been advanced. 1) The level of endogenous auxin may be too low due to inactivation of IAA by light. In some cases increased exogenous auxin concentration can substitute for darkness (Nanda et al., 1968). This is not the case with the species in question here, however, and other workers have also failed to replace the dark requirement with auxin (Pierik, 1969; 1970). 2) Phytochrome may participate in root formation. Furuya and Torrey (1964) presented evidence for a red / far red reversible control of lateral root initiation following auxin stimulation in isolated pea roots and Letouze and Beauchesne (1969) provided evidence that

red light is more efective than blue light in promotion of root initiation. On the other hand, Weis and Jaffe (1969) reported that blue light was more effective. These apparent conflicts in the literature may be due to the different starting material used i.e. shoots or callus. 3) Other substances involved in root initiation may be absent or diminished in light, for example, rooting cofactors or inhibitors. This aspect is discussed at greater length in Section 4.2. Galston (1948) found that asparagus stem tips repeatedly cultured in darkness, lost their capacity to root when IAA was supplied. He deduced from this experiment, that something essential for rooting was produced in light but that the actual root initiation process was inhibited by light. However, an additional problem with his experiment is the fact that the plant material underwent repeated subculture. This could have resulted in several additional deteriorative effects (see Section 4.3).

Root type in vitro

Root type did not vary with treatment but varied considerably with species. For example, Chaenomeles formed fleshy unbranched roots whereas the roots of Spiraea were thin with many branches. Descriptions

of other root systems after IBA application vary. Bowen et al. (1975) reported that cuttings of Pinus treated with IBA produced a finely divided root system, contrasting with that produced when NAA was supplied (thick, fleshy unbranched roots). On the other hand, Libby and Conkle (1966) suggested that IBA-induced roots were usually of larger diameter than those formed on control plants. In my experiments, differences with treatment were not observed, except that at high IBA concentrations, roots were shorter than at low concentrations.

All species except Spiraea had root hairs although the frequency of these was observed to be less than in soil-grown plants. In particular, Chaenomeles had very few. This may lead to decreased absorption when the plants are transferred to soil and thus reduce survival (see Section 4.3).

Roots of Cotoneaster did not penetrate the medium and roots grew in the air space. This may indicate a high oxygen requirement for root growth in this species. Roots of all other species penetrated the medium.

Activated charcoal and root initiation

Activated charcoal was incorporated in the nutrient medium to darken the medium. Colour density depended on charcoal concentration. It was found, however, that less root initiation occurred when charcoal was present than when it was absent in cultures exposed to light (16 hour photoperiod). Furthermore, low and high concentrations decreased rooting more than intermediate concentrations. Activated charcoal was therefore ineffective as a replacement for darkness.

Charcoal has been shown to increase rooting rate in several species, for example, strawberry (Damiano, 1978), Allium (Fridborg and Eriksson, 1975). It may adsorb inhibitory substances and growth regulators present in the medium (Reinert and Bajaj, 1977). Rooting could therefore be affected by this rather than by the darkening effect of the medium. Charcoal, in this case, appears to be removing auxin inhibitors as lower concentrations of IBA were necessary for rooting when the charcoal content increased.

Dynamics of in vitro root formation

In all species, the greatest number of roots was formed in the second and third weeks. This was the same as for mini cuttings rooted in greenhouse conditions. However, these shoots had not been pretreated with cytokinin as had the mini cuttings. Therefore, some factor in the in vitro environment other than cytokinin promotes rapid root initiation.

Number of roots formed varied with duration of exposure to IBA. Longer exposure periods increased the number of roots initiated but increase was less after the first week of culture. This may show that 1) most of the IBA in the medium is absorbed by the shoot in the first seven days, 2) sufficient IBA for root initiation is absorbed in the first seven days, and thereafter additional absorption of IBA does not affect root initiation to a great extent, or 3) the shoot can only absorb a limited quantity of IBA and the limit is nearly reached within seven days. It is unlikely, however, that the shoot has a limited capacity for IBA absorption as IBA would be continually deactivated in the shoot thus allowing additional uptake to occur. Alternatively, all suitable sites for root initiation might have been stimulated to produce roots within

seven days and thus additional auxin could not trigger additional root initiation.

4.2 THE ROLE OF ENDOGENOUS FACTORS ON ROOT FORMATION

The previous section demonstrated the importance of auxin in root initiation in most Ericaceous and Rosaceous species. However, differences in exogenous auxin requirement were observed between species. If control of rooting is by auxin concentration, this may indicate that either endogenous auxin content varies with species or that different species require different auxin content for root initiation.

Endogenous auxin content is known to vary from one part of the plant to another and with season. Therefore, the following factors were selected for examination in relation to root formation :- explant size, presence of axillary buds, presence of shoot apex, derivation of explant and seasonal variation. It is hypothesised that a study of rooting in relation to these factors will give implied information about the importance of endogenous auxin in root formation.

4.21 ROOT FORMATION IN STEM EXPLANTS DERIVING FROM DIFFERENT PARTS OF THE PLANT

Method

The species used were Prunus cerasifera and Spiraea 'Froebelii'. IBA was incorporated in the medium at 0.5, 1.0 or 5.0 mg l^{-1} . Incubation was in darkness for one week followed by light incubation treatment (b)(see section 4.11)(16 hour photoperiod) for three weeks.

Experiment 1 : Explant size

Shoot explants 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 or 25.0 mm in length were cultured. Root number was recorded at the end of a four week incubation period.

Experiment 2 : Presence of axillary buds

Each explant had 0, 1, 2, 3, 4, 5 or 6 axillary buds. Buds were removed from explants to obtain these bud numbers. Root number was recorded at the end of a four week culture period.

Experiment 3 : Presence of shoot apex

The terminal three millimetres of the shoot tip was either removed or left intact. Root number was recorded at the end of a four week culture period.

Experiment 4 : Derivation of explant

Explants were selected from the top, middle or base of the plant. Root number was recorded at the end of a four week culture period.

Results

Experiment 1 : Explant size

Fitted curves were plotted fro root number against explant size ($p<.001$ for fit of curve) (Figures 325 and 326). An analysis of variance (Table 114) showed a significant effect on root number due to explant size ($p<.001$), IBA concentration ($p<.001$), species ($p<.001$) and a significant interaction between these ($p<.001$). Thus, root number increased with explant size - most roots were formed in 25 mm explants in both species (Table 115).

Experiment 2 : Presence of axillary buds

Curves were plotted for Root number against bud number (Figures 327 and 328). An analysis of variance showed a significant effect on rooting due to bud number ($p<.001$), IBA concentration ($p<.001$) and species ($P<.001$) and a significant interaction between these ($p<.01$ - $p<.001$) (Table 116). Root number increased with bud number except in Spiraea at 5.0 mg l^{-1} IBA where bud number did not affect rooting. Thus, most roots were formed when 6 buds were present (Table 117).

Experiment 3 : Presence of shoot apex

Mean root number for explants with the apex present or absent is given in Table 118 for Prunus and Table 118 for Spiraea. More roots formed when the apex was present in both species. An analysis of variance (Tables 119 and 120) showed a significant effect on rooting due to presence of the apex ($p<.001$) and a significant effect due to IBA concentration ($p<.001$). The interaction between these was significant for Prunus ($p<.001$) but not for Spiraea.

Experiment 4 : Derivation of explant

Root number for explants form the top, middle and base of the plant is given in Table 121 for Prunus and Table 122 for Spiraea. Explants from the top of the plant formed more roots than those from the base. An analysis of variance showed showed a significant effect on root number due to derivation of explant ($p<.001$) and to IBA concentration ($p<.001$) for both species, and a significant interaction between these for Spiraea ($p<.001$) but no significant interaction for Prunus (Tables 123 and 124).

Figures 325 and 326.

Number of roots formed in four weeks incubation on medium containing IBA : effect of explant length.

Figures 327 and 328.

Mean number of roots formed in four weeks incubation on medium containing IBA : effect of bud number.

Prunus cerasifera 0.5 mg l^{-1} IBA $r = .8840$ ($p < .001$)

1.0 mg l^{-1} IBA $r = .9590$ ($p < .001$)

5.0 mg l^{-1} IBA $r = .9728$ ($p < .001$)

Spiraea 'Froebelii' 0.5 mg l^{-1} IBA $r = .9195$ ($p < .001$)

1.0 mg l^{-1} IBA $r = .9178$ ($p < .001$)

5.0 mg l^{-1} IBA $r = .3374$ (N.S.)

Fig 325. Prunus cerasifera.

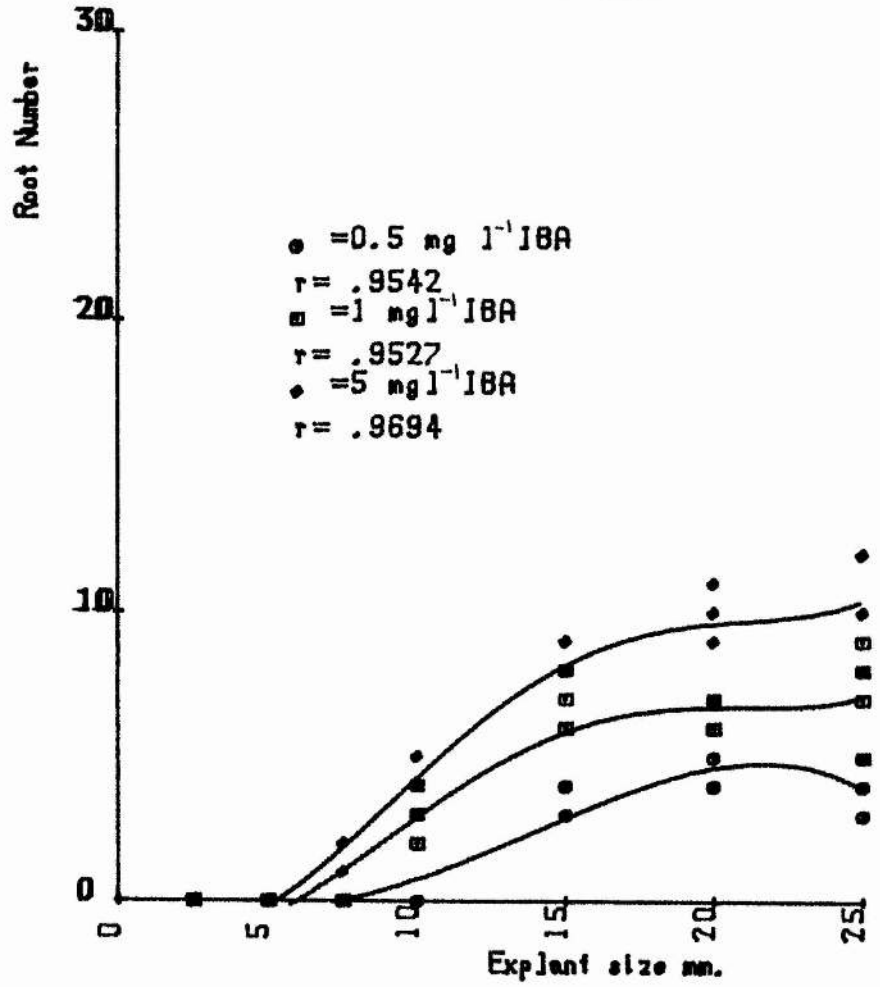


Fig 326. Spiraea 'Froebelii'.

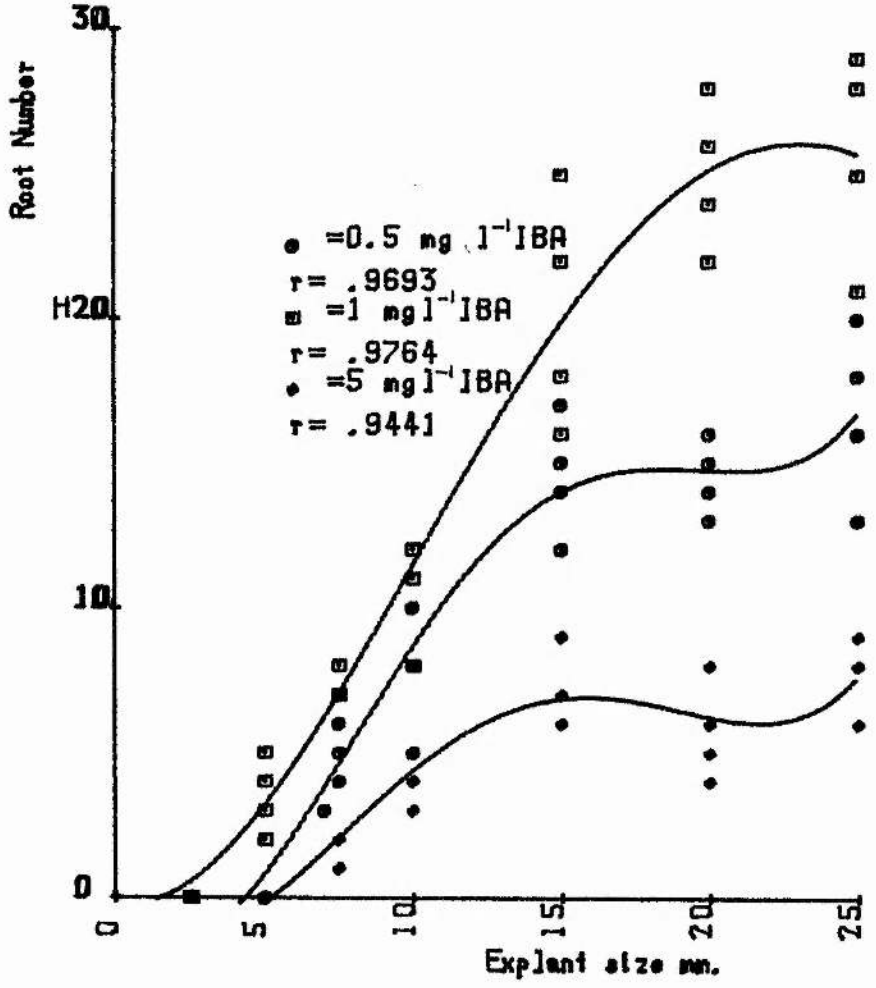


Fig 327. Prunus cerasifera.

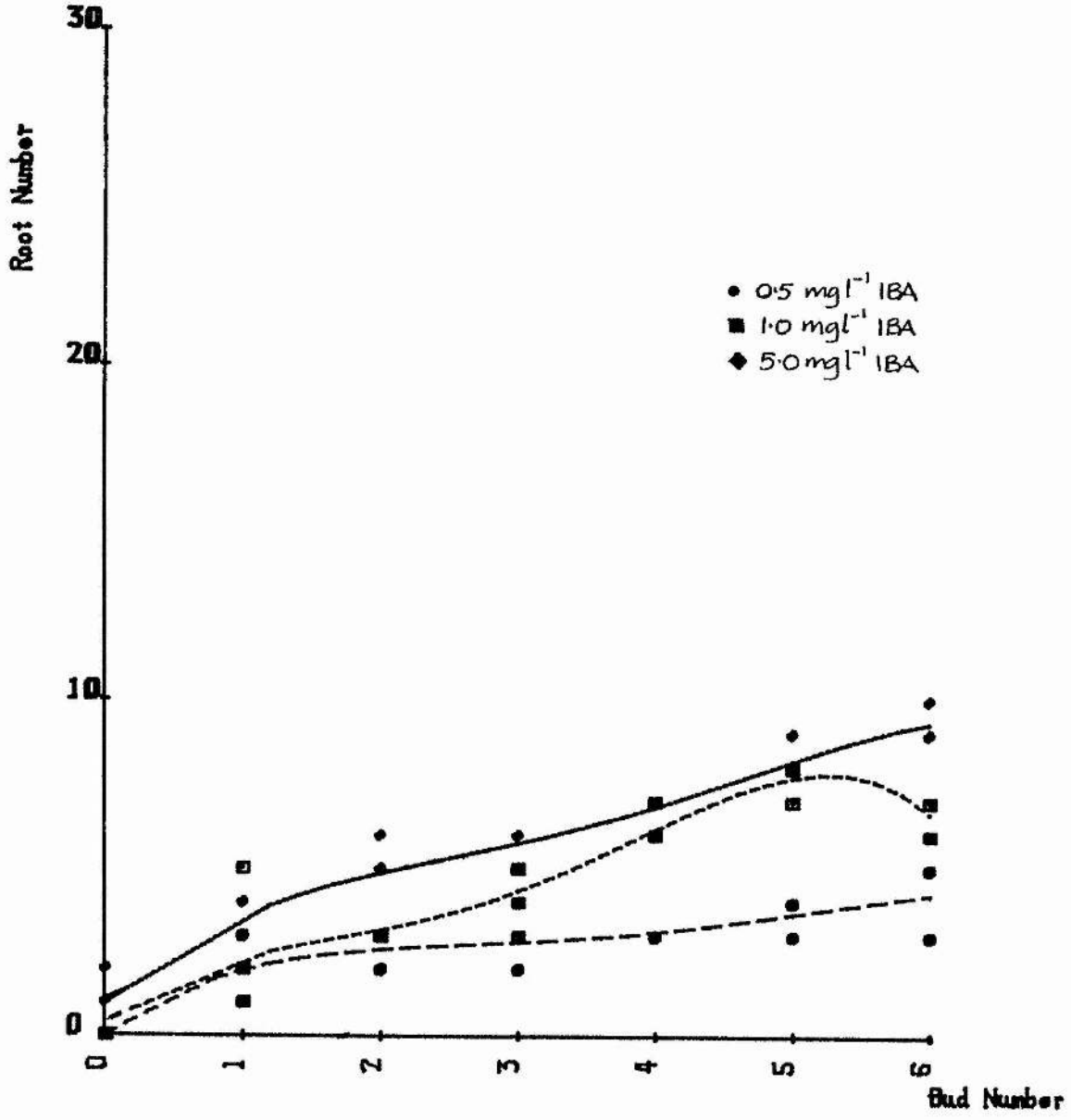


Fig 328. Spiraea 'Froebelli'.

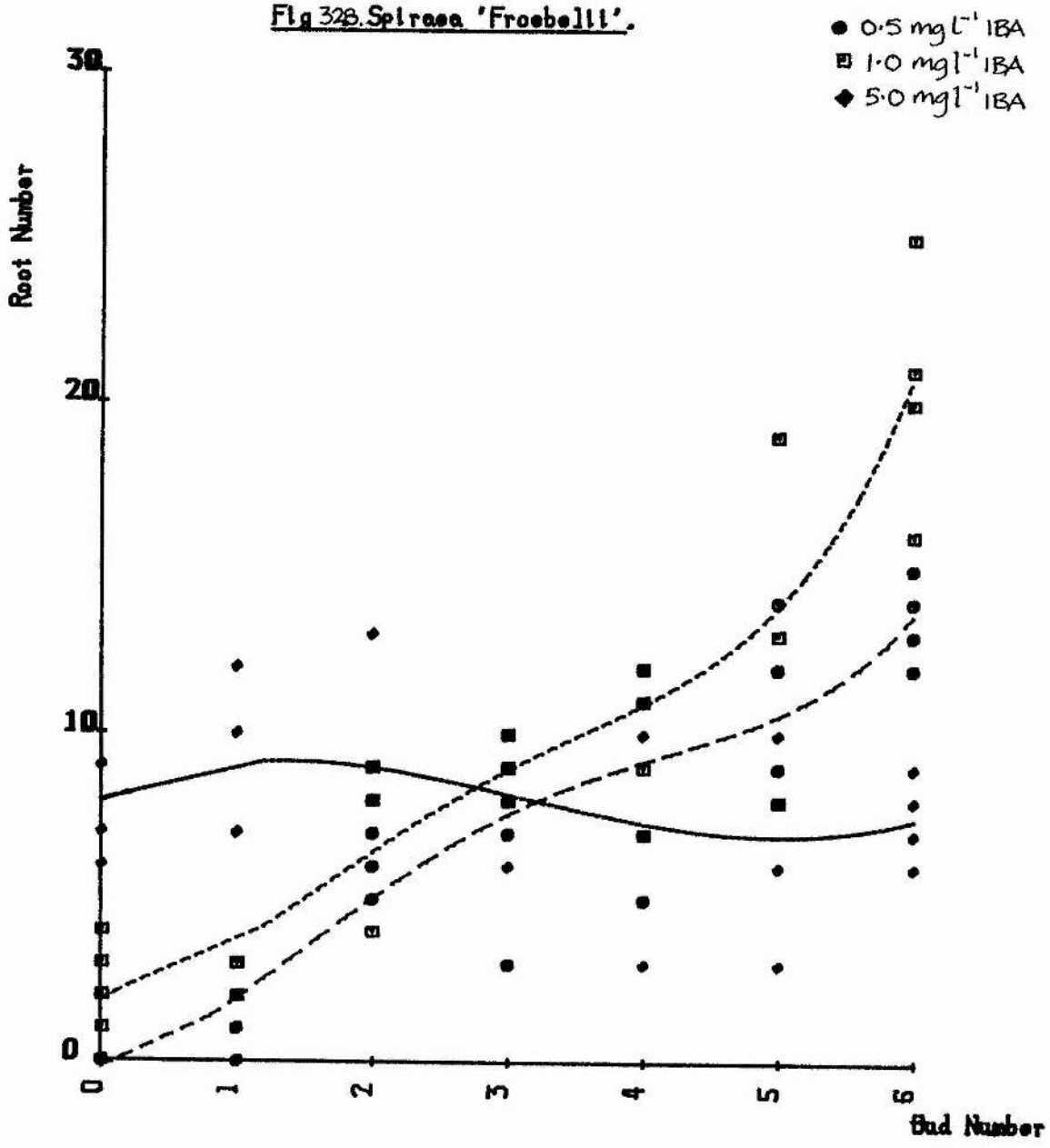


Table 114.

Analysis of variance for data presented in Figures 325 to 326.

SOURCE	S.S.	D.F.	M.S.	F	p
Size mm	148.379	6	24.730	1004.288	<.001
IBA conc.	5.804	2	2.0902	117.857	<.001
Species	21.744	1	21.745	883.063	<.001
Size * concentration	3.127	12	0.261	10.582	<.001
size * species	7.111	6	1.185	48.129	<.001
concentration * species	13.639	2	6.819	276.934	<.001
size * conc * species	6.362	12	0.530	24.065	<.001
Error	3.103	126	0.0246		
Total	209.269	167			

based on loge (y+1) transformation.

Table 115. Change in mean root number with explant size : in Spiraea 'Froebelii' and Prunus cerasifera.'.

Explant size mm	Spiraea 'Froebelii'	Prunus cerasifera
2.5	0f	0f
5.0	1.17e	0f
7.5	4.75d	0.33e
10.0	7.42c	2.33d
15.0	14.17b	6.42c
20.0	15.08b	6.58b
25.0	16.75a	7.52a

Means followed by different letters (within columns) are significantly different ($p<.05$).

Table 116.

Analysis of variance for data presented in Figures 327 to 328.

SOURCE	S.S.	D.F.	M.S.	F	p
Bud number	48.797	6	8.133	128.797	<.001
IBA conc.	7.607	2	3.804	60.239	<.001
Species	12.145	1	12.145	192.335	<.001
Bud * concentration	6.736	12	0.561	8.890	<.001
Bud * species	2.168	6	0.361	5.722	<.001
concentration * species	0.432	2	0.216	3.424	0.05
Bud * conc * species	8.766	12	0.730	2.861	<.01
Error	7.956	126	0.0631		
Total	94.607	167			

based on loge (y+1) transformation.

Table 117. Change in mean root number with explant bud count : in Spiraea 'Froebelii' and Prunus cerasifera.'.

Explant size mm	Spiraea 'Froebelii'	Prunus cerasifera
0	3.42e	0.25e
1	4.25d	2.58d
2	7.75c	3.58c
3	7.83c	4.33bc
4	8.83bc	5.33ab
5	10.83b	6.58a
6	13.83a	6.75a

Means followed by different letters (within columns) are significantly different ($p<.05$).

Table 118. Mean root number after 4 weeks incubation on medium containing IBA : +/- apex : Spiraea 'Froebelii'.

'.

IBA mg l^{-1}	-apex	+ apex	Mean
0.5	7.25	14.5	10.88b
1.0	10.00	20.25	15.13a
5.0	2.5	7.75	5.13c
Mean		14.17a	6.58b

Means followed by different letters (within columns or rows) are significantly different ($p<.05$).

Table 118b. Mean root number after 4 weeks incubation on medium containing IBA : +/- apex : Prunus cerasifera.

IBA mg l^{-1}	-apex	+ apex	Mean		
0.5	1.5	3.75	2.63c	3.0	4.88b
1.0	3.0	6.75	4.88b		
5.0	2.75	8.75	5.75a		
Mean		6.42a	4.42		

Means followed by different letters (within columns or rows) are significantly different ($p<.05$).

Table 119.

Analysis of variance for data given in Table 118 : Prunus cerasifera.

SOURCE	S.S.	D.F.	M.S.	F	p
+/- apex	96.000	1	96.000	172.800	<.001
IBA conc	41.583	2	20.792	37.425	<.001
Interaction	14.250	2	7.125	12.825	<.001
Error	10.000	18	0.556		
Total	161.833	23			

No transformation was used, however, if a $\log_e(y+1)$ transformation was used then the interaction was not significant.

Table 120.

Analysis of variance for data given in Table 118 : Spiraea 'Froebelii'.

SOURCE	S.S.	D.F.	M.S.	F	P
+/- apex	345.042	1	345.042	63.214	<.001
IBA conc	403.000	2	201.500	36.916	<.001
Interaction	25.333	2	12.667	2.321	N.S.
Error	98.250	18	5.458		
Total	871.625	23			

Table 121. Change in mean root number after 4 weeks incubation on medium containing IBA with origin of explant : Prunus cerasifera.

IBA mg l^{-1}	TOP	MIDDLE	BASE	Mean
0.5	3.75	3.0	1.25	2.67c
1.0	6.8	4.8	3.0	4.8b
5.0	8.8	8.3	4.0	7.0a
Mean	6.4a	5.3a	2.8b	4.8

Table 122. Change in mean root number after 4 weeks incubation on medium containing IBA with origin of explant : Spiraea 'Froebelii'.

IBA mg l^{-1}	TOP	MIDDLE	BASE	Mean
0.5	14.5	7.5	8.0	10.0b
1.0	20.3	14.8	9.0	14.7a
5.0	7.8	11.5	11.0	10.1b
Mean	14.2a	11.3b	9.3b	11.6

Means followed by different letters within a column or row are significantly different ($p<.05$).

Table 123.

Analysis of variance for data given in Table 121 :

Prunus cerasifera.

SOURCE	S.S.	D.F.	M.S.	F	p
origin	85.167	2	42.583	35.377	<.001
IBA conc	112.667	2	56.333	46.800	<.001
Interaction	10.667	4	2.667	2.215	N.S.
Error	32.500	27	1.204		
Total	241.000	35			

Table 124.

Analysis of variance for data given in Table 122 :

Spiraea 'Froebelii'.

SOURCE	S.S.	D.F.	M.S.	F	p
origin	142.166	2	71.083	9.730	<.001
IBA conc	171.167	2	85.583	11.715	<.001
Interaction	266.167	4	66.542	9.108	<.001
Error	197.250	27	7.306		
Total	776.750	35			

4.22 SEASONAL VARIATION

Method

Species used were Spiraea 'Froebelii' and Prunus cerasifera. IBA was incorporated in the nutrient medium at 0.5, 1.0 and 5.0 mg l^{-1}. Shoots were cultured at monthly intervals during the spring, summer and autumn from stock plants which were kept out of doors. Root number was recorded after four weeks.

A greenhouse experiment was conducted simultaneously. Shoots were placed in peat : perlite medium (1:1 by volume) in the greenhouse at monthly intervals during the spring, summer and autumn. Shoots were treated with 1) no auxin or 2) auxin quick dip as detailed previously. Number of rooted cuttings was recorded after four weeks.

Results

Root number varied with month of explant culture and was greatest in May and June in both species (Figures 329 and 330; Table 126). An analysis of

variance (Table 125) showed a significant effect on root formation of month of culture ($p<.001$), IBA concentration ($p<.001$), species ($p<.001$) and a significant interaction between these ($p<.001$).

A similar pattern of root formation with season was recorded in the greenhouse experiment (Figures 331 and 332). An analysis of variance (Table 127) showed a significant effect due to season ($p<.001$), IBA treatment ($p<.05$), species ($p<.001$) and a significant interaction between these ($p<.01$ to $p<.001$). Most root formation occurred in May and June and another lower peak of root formation occurred in September (Table 128). A rise in rooting in September was not noted in the _in vitro_ experiment. From July to September, in _Spiraea_, IBA decreased rooting, whereas in March, April and October, IBA increased rooting. In _Prunus_, rooting was enhanced by IBA in all months.

Figures 329 and 330.

Mean root number formed in a four week incubation period on medium containing IBA : effect of month of culture.

Figures 331 and 332.

Number of stem cuttings which had rooted after four weeks: effect of month of propagation.

Fig 329 Prunus cerasifera.

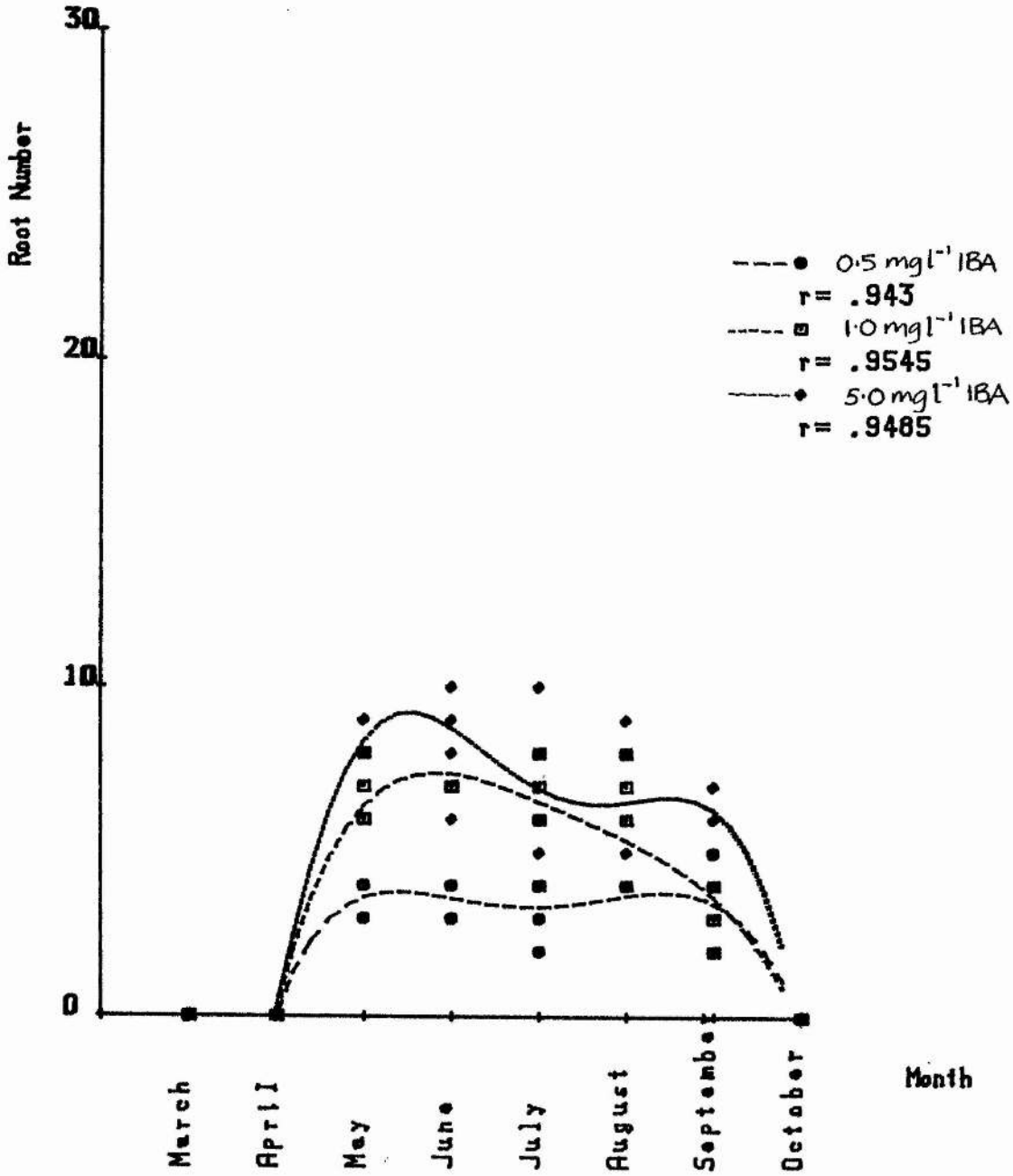


Fig 330. Spiraea 'Froebelii'.

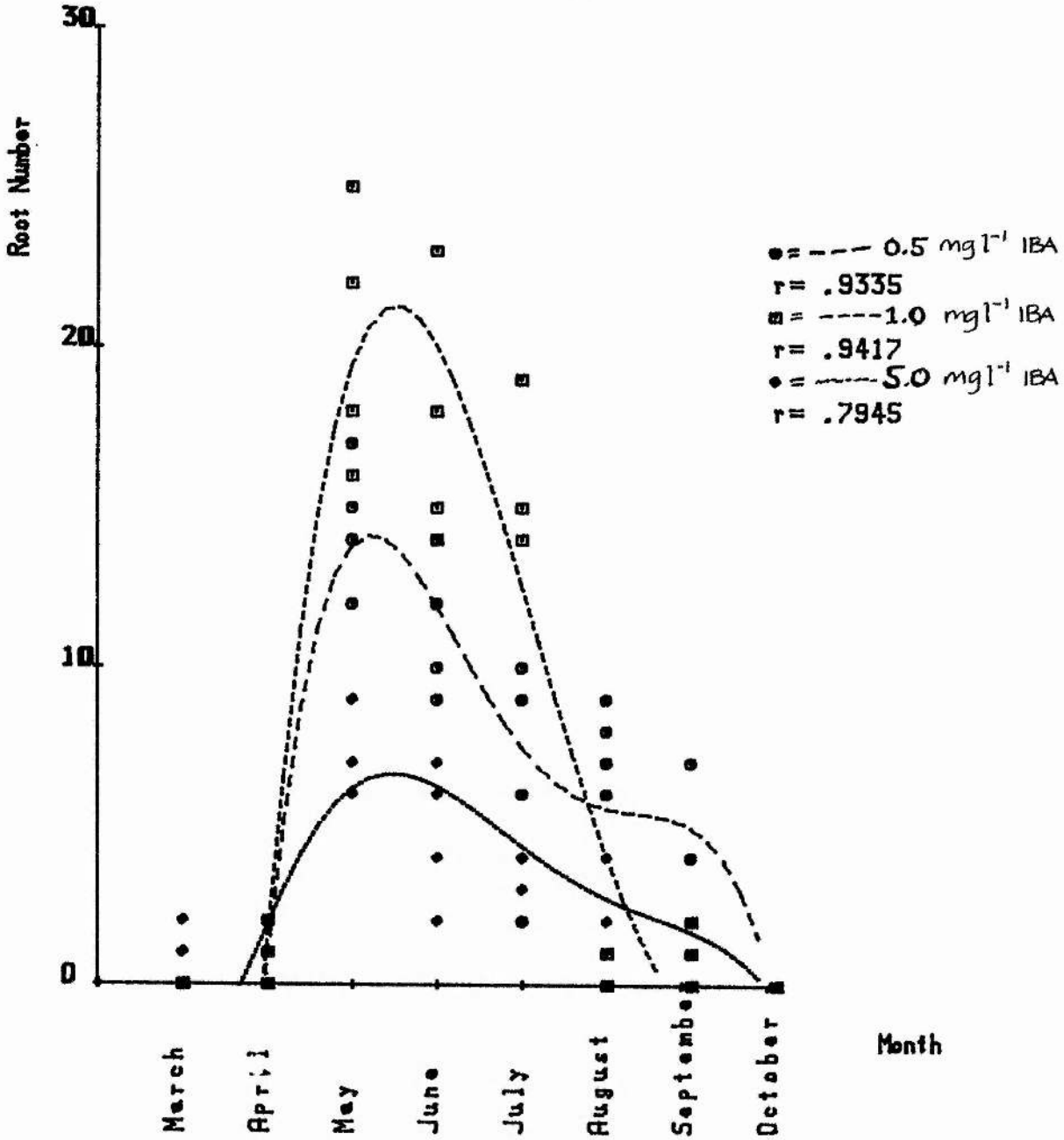


Fig 331. Prunus cerasifera.

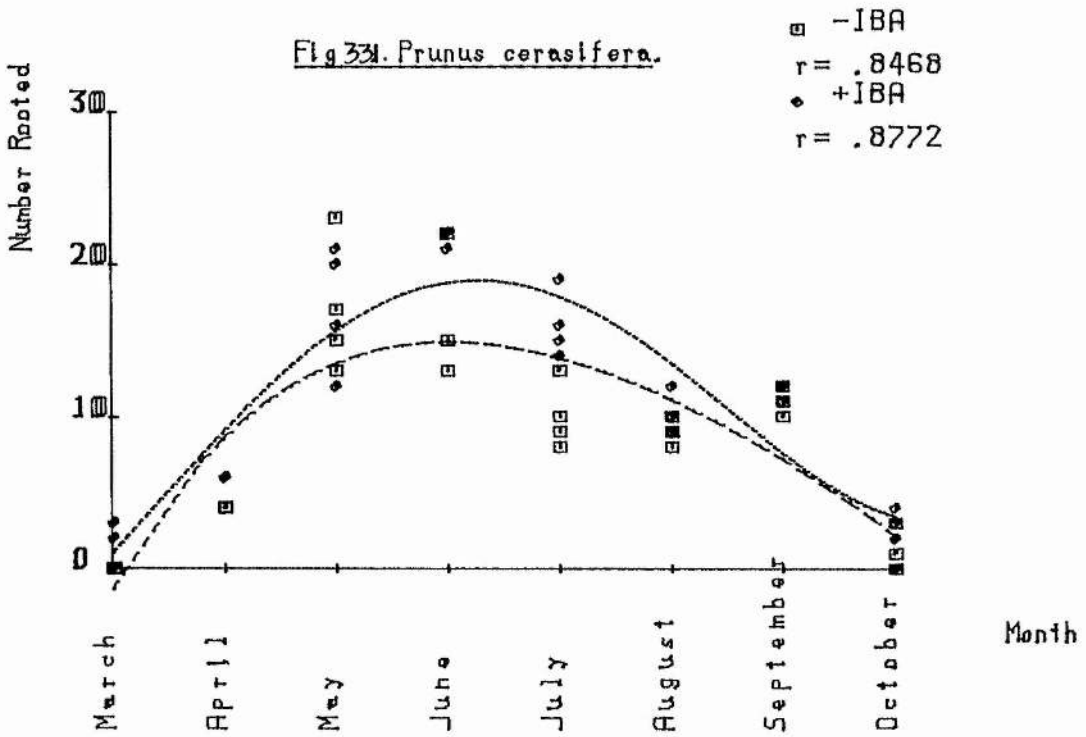


Fig 332. Spiraea 'Froebellii'.

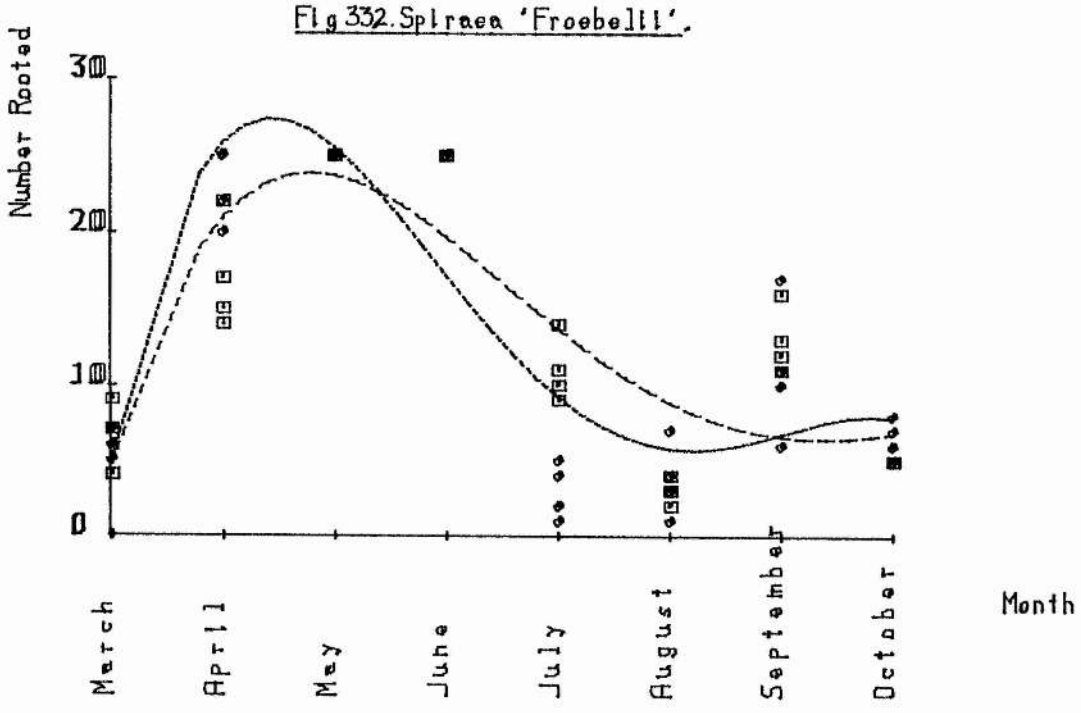


Table 125.

Analysis of variance for data presented in Figures 329 to 330.

SOURCE	S.S.	D.F.	M.S.	F	p
Season	2845.146	7	406.449	173.162	<.001
IBA conc.	103.198	2	51.599	21.983	<.001
Species	130.021	1	130.021	55.393	<.001
Season * concentration	429.635	14	30.688	13.074	<.001
Season * species	506.979	7	72.426	30.856	<.001
concentration * species	266.948	2	133.473	56.865	0.001
Season * conc * species	518.052	14	37.003	15.428	<.001
Error	338.000	144	2.347		
Total	5137.979	191			

When a loge (y+1) transformation was used the effect of IBA concentration was not significant.

Table 126. Mean root number variation with season : in Spiraea 'Froebelii' and Prunus cerasifera.'.

Month	Mean root number	
	Spiraea 'Froebelii'	Prunus cerasifera
March	0.21	0
April	0.41	0
May	2.64	1.96
June	2.36	1.93
July	2.12	1.82
August	1.40	1.85
September	0.61	1.58
October	0	0

L.S.D. = 0.21

Table 127.

Analysis of variance for data presented in Figures 331 to 332.

SOURCE	S.S.	D.F.	M.S.	F	p
Month	58.045	7	8.292	99.073	<.001
IBA	0.436	1	0.436	5.211	<.05
Species	3.873	1	3.873	46.280	<.001
Month * IBA	2.118	7	0.303	3.615	<.01
Month * species	22.092	7	3.156	37.707	<.001
IBA * species	1.425	1	1.425	17.024	0.001
Month * IBA * species	2.252	7	0.322	37.707	<.001
Error	8.035	96	0.0837		
Total	98.275	127			

based on loge (y+1) transformation.

Arcsin $\sqrt{p}$ calculation gave similar results.

Table 128. Percentage rooting of cuttings with season : in Spiraea 'Froebelii' and Prunus cerasifera.'.

Month	Mean root number	
	Spiraea 'Froebelii'	Prunus cerasifera
March	24c	4e
April	80a	20c
May	100a	68a
June	100a	80a
July	28c	52b
August	13d	38b
September	48b	45b
October	22c	6d

Means followed by a different letter (within a column) are significantly different ($p<.05$), based on an arcsin p transformation.

4.23 DISCUSSION

Explant size

Fewer roots were formed on very small explants. Others have noted that rooting decreases with size of cutting (Komissarov, 1969) but Pierik and Steegmans (1975) noted that length of explants (1.0, 1.5 or 2.0 cm) did not affect rhizogenesis in Rhododendron.

Several reasons could account for the observed difference in rooting between long and short explants. 1) Surface area in contact with the nutrient medium is small and therefore, absorption of sucrose and minerals may be less than in larger explants. Growth and differentiation might therefore be slower. 2) Endogenous auxin content in a small explant is less than in a large explant. However, it is unlikely that auxin is limiting as higher exogenous auxin concentrations failed to promote rooting in small explants. 3) Some other endogenous factor is not present in sufficient quantity to allow more than a few roots to form.

Only a slight increase in root number was observed with increase in explant size from 15 to 25 mm. The hypothesis (2-above) that there is insufficient auxin

available in small explants for increased root initiation to occur was disproved, as higher auxin concentrations did not stimulate any additional root formation. Roots always arose at the base of the shoot regardless of length. Therefore, the number of root initiation sites in the basal few millimetres may limit the number of roots which can form.

Presence of buds

More roots were initiated when buds were present than when buds were removed. Therefore, buds promote root formation in the two species tested. Other workers have reported promotion of rooting by buds (Van der Lek,1924, 1934; Selim, 1956; Fadl and Hartmann, 1967). Selim (1956) showed that buds on Perilla cuttings accounted for 15% of the rooting response, and Fadl and Hartmann (1967) showed that buds on 'Old Home' pear promoted root initiation from July to October.

Haissig (1972) showed that a ring of TIBA (tri-iodobenzoic acid) applied between the second and third nodes in cuttings of Salix, reduced the level of extractable auxin below the ring by 50%. TIBA blocked auxin transport from the buds. The number of root primordia initiated was also reduced by 50% and

the number of cells per primordium by 75%. The importance auxin in buds was thus demonstrated. Wetmore and Rier (1963) showed that differentiation of vascular tissue in callus of Syringa was stimulated by grafting a bud to the callus. Furthermore, they demonstrated that the bud could be replaced by IAA and sucrose with the same result. This work shows the importance of bud endogenous auxin content in differentiation.

In my work, although interaction with other substances cannot be excluded from consideration, endogenous auxin in buds appears to be important in determining root initiation. When buds were removed, a higher exogenous auxin stimulated root initiation. Exogenous auxin, however, could not completely replace the effect of the buds. As sucrose was supplied in the nutrient medium, it is unlikely that the lack of sucrose from the buds is a limiting factor when buds are removed. The role of sucrose in differentiation is investigated further in Chapter VI.

Presence of shoot apex

When the shoot apex was removed, root number decreased and was not significantly increased by

exogenous auxin. The shoot apex is a site of auxin biosynthesis but as exogenous auxin did not replace the apex effect, the promotive effect of the apex is not due solely to auxin.

Biron and Halevy (1973) reported that removal of the growing point of dahlia cuttings improves rooting. They suggested that this occurs because the apex competes for nutrients required for root initiation. In my experiment, nutrients were supplied in the medium and therefore nutrients are unlikely to be limiting. However, root initiation is frequently favoured by low nutrient levels. The medium used contained a high level of nutrients and therefore, competition by the apex for nutrients could reduce the level of nutrients available at root initiation sites and thus enhance rooting.

Derivation of explant

Explants from the top of the mother plant formed a greater number of roots than those from the base. Increased exogenous auxin concentration enhanced root formation in base cuttings to such an extent that root number equalled that of top cuttings. This implies that endogenous auxin level is lower at the base of the

plant than at the top and that endogenous auxin does make a significant contribution to the rooting response in this instance.

Others have reported the reverse of my results when cuttings were rooted in greenhouse conditions. Root formation was greater in cuttings taken from the base (Komissarov, 1969; Hartmann and Kester, 1975). This may vary with season and with condition of the cuttings. Shoot explants used in my experiments were actively growing. If the authors quoted above used woody cuttings from the base of the plant (not stated), then a different effect on root formation might have resulted.

Seasonal variation

Root number was greatest in explants collected in spring and early summer. Other workers have noted similar results, for example, root formation in vitro in other cultivars of Rhododendron only took place in young soft shoots (Pierik and Steegmans, 1975).

Inhibition of rooting at certain times of year has been related to changes in parts of the plant, particularly lateral buds. Dormant buds have been

shown to be inhibitory to rooting (Van der Lek, 1924, 1934) but the presence of buds in summer can promote rooting (Fadl and Hartmann (1967). Fadl and Hartmann showed that in 'Old Home' pear, an easy-to-root cultivar, the presence of buds promoted rooting from July to October but that buds had no effect from November to March. However, in 'Bartlett' pear, a difficult-to-root cultivar, buds were inhibitory at all times of year. On the other hand, in a comparison of rooting of softwood cuttings of Populus which had either an active growing point or a dormant terminal resting bud, Smith and Wareing (1972) found that the difference in rooting response was relatively small. Reports in the literature therefore vary but it seems that the effects of lateral and terminal buds on rooting may be different and species-dependent. In the current experiment, buds were left intact, and therefore, could have had an effect on rooting. Both species exhibited the same seasonal rooting pattern.

The effect of terminal buds on rooting has been correlated with change in auxin content of the buds. When the growing point ceases to produce actively growing young leaves, its level of auxin production falls (Wareing, 1973). However, auxin may not be

deactivated as rapidly as in actively growing shoot tips - Smith and Wareing (1972a) found that the auxin content of dormant leafy cuttings was not significantly different from that in actively growing cuttings. However, auxin content is affected by chilling (normally experienced in winter); and Smith and Wareing (1972b) demonstrated higher levels of auxins in chilled than in unchilled poplar cuttings.

Changes in endogenous content of other growth regulators have also been reported as a result of chilling : cytokinins increase in shoots of Populus (Hewett and Wareing, 1973) and gibberellins in other species increase (Wareing and Saunders, 1971). Abscisic acid content also changes with season (Bausher, 1976) : a rise in free ABA levels occurs in autumn and a gradual decline occurs during winter in Fagus (Wright, 1975). Gibberellin and cytokinin rise during winter but decline in concentration in spring (Alvim et al., 1976; Moore, 1979).

Another change which occurs during winter is a change in photoperiod and this has also been shown to affect rooting in some species. In Rhododendron cultivars, cuttings from stock plants grown under long days rooted better than those from short photoperiod

(Barba and Pokorny, 1975). This effect may be due to maintainance of active growth in the stock plants. In my experiment, stock plants were kept outside throughout the year and therefore, active growth ceased in autumn. Reduction of rooting in autumn may therefore be due to reduced daylength or temperature.

In both the greenhouse and the in vitro experiment, a 16 hour daylength was given at all times of year but reduction of rooting at certain times of year still occurred. Other workers have reported varying results when extended photoperiod treatment is given to cuttings; Baker and Link (1963) found little effect of photoperiod at times of year when rooting was easy but extended daylengths could improve root initiation and root number in a few subjects when rooting became more difficult, and French (1983) showed that adventitious root initiation was enhanced by increased photoperiod in some Rhododendron cultivars but not in others. On the other hand, Lanphear and Meahl (1961) found a depressant effect of extended photoperiod with Juniperus and Taxus when cuttings were rooted in winter. However, they found no effect of photoperiod in other species including Pieris, Rhododendron and Pyracantha. This work on

Ericaceous and Rosaceous species supports my results.

Control of rooting by photoperiod may be hormonally controlled. IAA levels can be increased by long photoperiods in both stem tissue (Nitsch and Nitsch, 1959; Wodzicki, 1964) and in leaf tissue (Digby and Wareing, 1966).

The above discussion shows that decline in rooting in autumn and winter may be due to change in auxin or other growth regulator content. However, it does not explain the decrease in rooting observed in cuttings in July and August prior to another increase in September. This decrease was observed in stem cuttings of both species tested.

Lee et al. (1969) working with Rhododendron cultivars found that a rooting inhibitor present in leaves and stems in July disappeared in September but reappeared in November. A similar inhibitor could be responsible for the results observed in my experiment.

However, the effect of the inhibitor (Lee et al., 1969) was found to be less important than change in rooting cofactors. (These factors are auxin synergists and are principally phenolic compounds (Hess, 1961).) There was an increase in cofactors in September corresponding with increased rooting in that month,

followed by a decreased in November corresponding with decline in rooting. Rooting cofactors have been found in other species, for example, Chrysanthemum, Hibiscus and Camellia, and were related to rooting ability (Hess 1964; Richards, 1964).

Hess (1961) suggested that the presence of four rooting cofactors in extracts obtained from Hedera helix cuttings was responsible for its high rooting capacity. Rooting cofactors occur in easy-to-root cuttings of some genera but are present in smaller amounts or absent in difficult ones (Fadl and Hartmann, 1967). The work of Lee et al. (1969) supports this. They found that in Rhododendron cultivars, the highest levels of four rooting cofactors in any season were found in stem and leaf tissue in easy-to-root 'Cunningham's White', lower levels in 'English Roseum' and lowest levels in 'Dr. D.C. Dresselhuys', a notoriously difficult cultivar to root. However, Lanphear and Meahl (1963) found no correlation between rooting cofactor activity and season of rooting in Juniperus and Taxus.

Jones and Hatfield (1976) cultured M7 apple in vitro with phloroglucinol and phloretic acid (breakdown products of phloridzin), and found that root

number was doubled. Phloridzin itself, caffeic acid, catechol and pyrogallol did not significantly increase rooting. Phloroglucinol and IBA also acted synergistically to promote rooting in Rubus but not in strawberry (James, 1979). Relating the molecular structure of a phenolic to its effectiveness as an auxin synergist has not led to any clear conclusions (Hess, 1964b; Basu et al., 1969). It has been suggested that the enhanced rooting results from these compounds inhibiting IAA oxidase activity, thus preventing the destruction of auxin (Basu et al., 1969). However, with apple shoots, phloroglucinol stimulated rooting in the presence of NAA and IBA which are not easily oxidized (Donoho et al., 1962; Kenten, 1955).

Thus, phenolic compounds may be responsible for change in rooting response with season. The interesting observation was made, however, that there was no rise in root formation in September in vitro. As the principle difference between shoots cultured in vitro and cuttings cultured in the greenhouse was nutrient status of the medium, it is probable that mineral status is affecting the rooting response.

Summary

This section investigated the importance of endogenous auxin content in root initiation. Supportive evidence for endogenous auxin contribution to the root initiation response was found. Exogenous auxin, applied to shoots taken from the base of the plant, could replace the promotive effect of the upper part of the plant, and applied to shoots with no buds, could partially replace the effect of buds. However, exogenous auxin did not replace the promotive effect of the shoot apex, and seasonal differences could not be overcome by additional auxin.

Therefore, although endogenous auxin plays a significant role in root formation, the results of the experiments indicate that other endogenous factors are also involved in determining the rooting response. Other factors which may be important are cytokinins, gibberellins, abscisic acid and phenols. Nutrient availability may also be critical.

4.3 RHIZOGENESIS AFTER REPEATED SHOOT SUBCULTURE

Repeated subculture results in changes in shoot morphogenesis (Section 3.3). Section 4.2 demonstrated that endogenous factors have a similar effect on root formation as on shoot formation. Therefore, it is hypothesised that an increase in rhizogenesis will occur when an increase in caulogenesis occurs and that decreased rhizogenesis will result from shoots with low caulogenetic potential. This section tests this hypothesis.

Treatment of subcultured shoots with gibberellin, auxin, cytokinin and reduced illumination resulted in a change in caulogenetic potential (Section 3.3). Shoots treated similarly were examined in this section for rhizogenetic potential to determine whether potential for root formation is controlled by the same factors as shoot formation.

4.31 ROOT FORMATION AFTER REPEATED CYTOKININ TREATMENTS

Method

Experiment 1.

The following species were used in the experiment :- *Chaenomeles japonica*, *Crataegus brachyacantha*, *Potentilla* 'Coronation Triumph', *Potentilla* 'Sutter's Gold', *Prunus* cerasifera, *Prunus tomentosa* and *Spiraea* 'Froebelii'.

Shoots were cultured on nutrient medium containing IBA at the following concentrations:- 0, 0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg l^{-1}. Generation 1 consisted of shoot explants taken directly from the stock plant in May. These had therfore not received any exogenous cytokinin. Thereafter, shoots were selected every four weeks from the treatment which had formed the greatest number of shoots during the previous culture period on medium containing BA (as detailed in Section 3.31). A total of 10 generations of shoots were examined for rooting potential.

Root number was recorded at the end of a four

week incubation period consisting of one week in darkness followed by three weeks of light incubation (16 hour photoperiod). Presence of callus was also recorded.

Experiment 2.

The species used for this experiment was Spiraea 'Froebelii'. Conditions of the experiment were the same as described in Experiment 1 above. Shoots previously cultured on medium containing 2iP were cultured on medium containing IBA at 0, 0.5, 1.0 or 2.5 mg l^{-1} for 10 generations.

Experiment 3.

Rooted shoots (complete plants), formed from each generation of Spiraea 'Froebelii' in Experiments 1 and 3 above, were potted in a peat / perlite (1:1) medium, covered with polythene and transferred to the greenhouse. Survival of the plants was recorded after two weeks.

Results

Experiment 1.

Root number declined with propagative generation in all species (Figures 333 to 338) except Chaenomeles (Table 129) in which there was no significant change in root number with generation (Table 130).

An increasing incidence of callus was noted in later generations. Callus was yellow / brown.

Experiment 2.

A decline in rooting potential was observed with propagative generation when shoots were repeatedly cultured on medium containing 2iP (Figure 339). However, a comparison of regressions showed that the decline was not as rapid as on medium containing BA (Figure 339). Callus formation was minimal.

Experiment 3.

The number of plants surviving decreased with number of previous subcultures from generations 6 to 10 (Table 131). No significant difference in survival in generations 1 to 5 between BA and 2iP

pretreated plants. However, 2iP treated plants survived significantly ($p<.01$) better than BA treated plants in generations 6 to 10 (Table 132).

Figures 333 to 338.

Number of roots formed in four week incubation periods on medium containing IBA : shoots previously subcultured on media containing BA.

Figure 339.

Decrease in root number (after incubation on medium containing IBA for four weeks) with propagative generation : comparison between repeated subculture on BA and 2iP medium.

Comparison of regression coefficients over 3 best concentrations.

BA r = -.8413
b = -1.8389
2iP r = -.6054
b = -.7667
$p < .001$

Fig 333. Crataegus brachyacantha.

y=62.26-6.69X1

R2= .9824

r= .9911

S.E.= 2.868

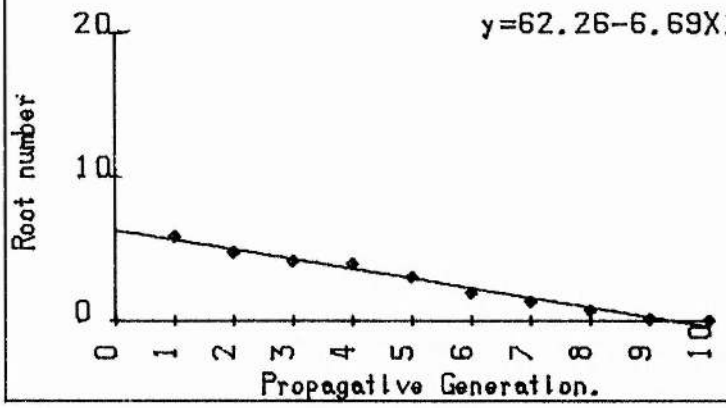


Fig 334. Potentilla fruticosa.

y=112.99-11.79X1

R2= .9489

r= .9741

S.E.= 8.772

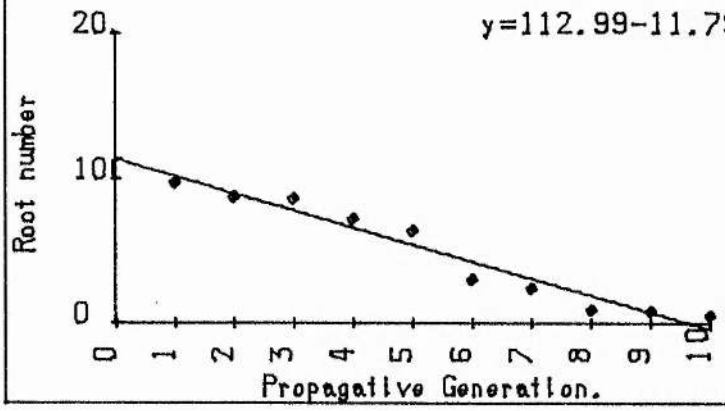


Fig 335. Potentilla 'Sutter's Gold'.

y=114.06-10.18X1

R2= .8995

r= .9484

S.E.= 10.922

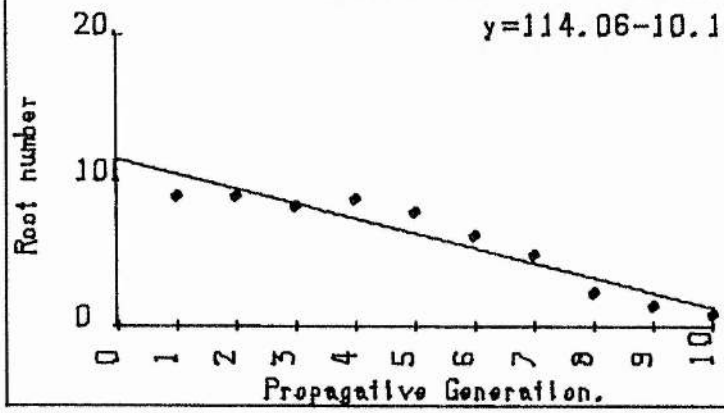


Fig 336. Prunus cerasifera.

y=110.46-11.02X1

R2= .9716

r= .9856

S.E.= 6.045

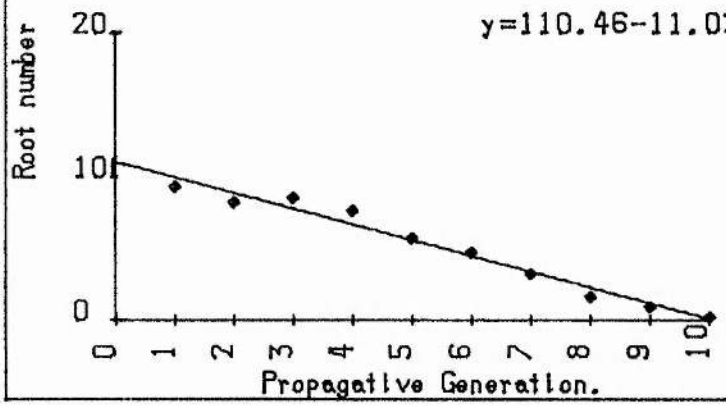


Fig 337. Prunus tomentosa.

y=18.39-2.06X1

R2= .8686

r= .932

S.E.= 2.565

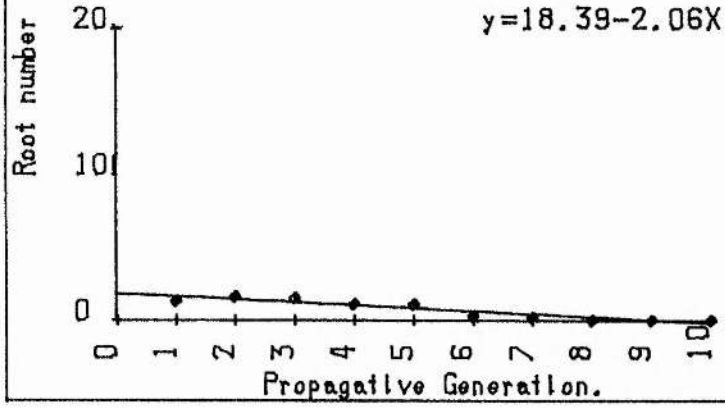


Fig 338. Spiraea 'Froebelii'.

y=209.19-22.31X1

R2= .9561

r= .9778

S.E.= 15.34

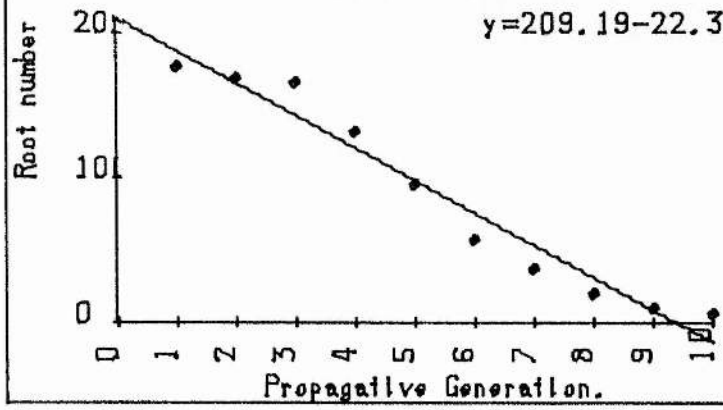


Fig 339. Spiraea 'Froebelii'.

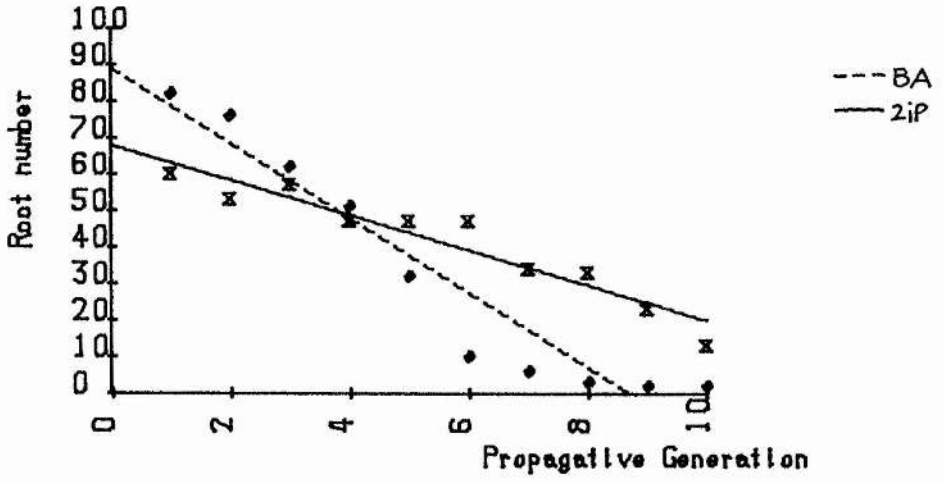


Table 129.

Change in mean root number with propagative generation and IBA concentration : Chaenomeles japonica.

Generation	IBA mg l^{-1}			
	2.5	5.0	10.0	20.0
1	1	6	5	0
2	1	8	6	2
3	4	6	7	4
4	4	8	6	4
5	2	3	5	4
6	6	4	7	6
7	6	4	4	4
8	1	3	4	4
9	4	3	4	0
10	3	4	5	0

Table 130.

Analysis of change in root number with propagative generation : Chaenomeles japonica.

GENERATION	1	2	3	4	5	6	7	8	9	10
ROOT No.	12	17	21	22	14	23	18	12	11	12
EXPECTED	16.2									

$\chi^2 = 11.83$ (N.S.)

Table 131.

Mean percentage survival from different propagative generations : Spiraea 'Froebelii'.

Generation	2iP	BA
1	100	100
2	100	100
3	75	100
4	100	100
5	75	100
6	25	100
7	25	50
8	0	75
9	0	0
10	0	25

Table 132.

Analysis of survival with propagative generation : Chaenomeles japonica.

	GENERATION		
	Percentage survival		
Treatment	1-5	5-10	p
BA	90	10	<.000001
2iP	100	50	<.0005
p	N.S.	<.01	

Quoted probabilities relate to difference of values in adjacent row or column (based on exact probability test). Results from the two treatments to be compared are pooled. An assumption of no difference is made and the exact probability test applied.

4.32 ROOT FORM ATION AFTER TREATMENT WITH GIBBERELLIN, AUXIN OR CYTOKININ

GA, IBA and 2iP were shown in Section 3.32 to promote shoot elongation. However, there was no parallel increase in shoot initiation. This section investigates whether prior treatment with GA, IBA or 2iP affects root formation.

Method

Ninth generation shoots of Spiraea 'Froebelii' which had been treated with GA, IBA or 2iP as described in Section 3.32 were cultured on nutrient medium containing IBA at the following concentrations :- 0, 0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg l^{-1}.

Root number was recorded at the end of a four week culture period as described in Section 4.31.

Results

Presence of GA in the medium at concentrations of 10 mg l^{-1} or greater, completely inhibited rooting (Table 133), while no significant increase in rooting was shown at lower GA concentrations (Table 134).

Root number after IBA pretreatment is given in Table 135. A χ^2 test showed that there was a significant interaction between IBA treatment in the previous generation and root initiation ($p<.001$) (Table 136). IBA at 0.5 mg l^{-1} stimulated root formation in the current experiment ($p<.01$).

Root number after 2iP treatment in the previous generation is given in Table 137. There was no significant difference in rooting between treatments (Table 128).

Table 133.

Change in mean root number with GA treatment of previous generation : Spiraea 'Froebelii'.

IBA mg l^{-1}	GA mg l^{-1}				
	0	0.5	5	10	50
0	0	0	0	0	0
0.5	0	0	0	0	0
1.0	0.5	0	0	0	0
2.5	0.5	0.5	0.75	0	0
5.0	0.25	0	0.25	0	0
10.0	0.25	0.5	1.0	0	0
20.0	0	0	0	0	0

Table 134.

Analysis of change in root number with GA application : Spiraea 'Froebelii'.

GA mg l^{-1}	Root number
0	0.214a
0.5	0.179a
5.0	0.250a
10.0	0b
20.0	0b

values followed by different letters are significantly different ($p<.05$).

Table 135.

Change in mean root number with IBA treatment of previous generation : Spiraea 'Froebelii'.

IBA mg l^{-1}	IBA mg l^{-1}			
	0	0.5	5	10
0	0	1.0	0	0
0.5	0	1.0	0	0
1.0	0.5	0.75	0	0
2.5	0.5	0.75	0	0
5.0	0.25	0.5	1.0	0
10.0	0.25	0.25	0.25	0.5
20.0	0	0.5	0.25	0.25

Table 136.

Analysis of change in root number with IBA application in previous generation : Spiraea 'Froebelii'.

	Root number		
	IBA mg l^{-1}		
Observed	6	19	3
Expected	8.5		

χ^2=18 ($p<.001$)

Table 137.

Change in mean root number with 2iP treatment of previous generation : Spiraea 'Froebelii'.

IBA mg l^{-1}	2iP mg l^{-1}				
	0	5	10	15	20
0	0	0	0	0	0
0.5	0	0	0	0	0
1.0	0.5	0	0	0	0
2.5	0.5	0.25	0.5	1.0	1.0
5.0	0.25	0.25	1.0	1.25	0.75
10.0	0.25	1.0	1.0	0.75	0.5
20.0	0	0	0	0.5	0

Table 138.

Analysis of change in root number with 2iP application in previous generation : Spiraea 'Froebelii'.

	Root number			
	2iP mg l^{-1}			
Observed	6	6	14	9
Expected	9			

$\chi^2 = 4.89$ (N.S.)

4.33 ROOT FORMATION AFTER REDUCED ILLUMINATION TREATMENT

Section 3.33 demonstrated that shoot length increased under conditions of reduced light intensity and / or photoperiod. This increase was, however, paralleled by a decrease in shoot formation. This section investigates root initiation after reduced light intensity and / or reduced photoperiod treatment in the previous generation.

Method

Ninth generation shoots of Spiraea 'Froebelii' which had undergone illumination treatments (Section 3.33) were cultured on nutrient medium containing IBA at 0, 0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg l^{-1}.

Root number was recorded at the end of a four week culture period as described in Section 4.31.

Results

Results are given in Table 139. Duration and intensity of light significantly affected root number ($p<.01$) (Table 140). Low light intensity and short days gave the highest root number and high light intensity and long days gave the lowest root number. However, there was no significant difference in root number with light intensity when results were pooled but a significant ($p<.001$) difference in root number with photoperiod was demonstrated (Table 141).

Table 139.

Change in mean root number with illumination treatment of previous generation : Spiraea 'Froebelii'.

IBA mg 1	Illumination			
	High		Low	
	16 hours	8 hours	16 hours	8 hours
0	0	0	0	0
0.5	0	0	1.0	1.0
1.0	0.5	0.5	1.5	0.75
2.5	0.5	1.0	1.25	2.0
5.0	0.25	1.25	1.25	1.75
10.0	0.25	1.25	1.0	1.5
20.0	0	0	0	0

Table 140.

Analysis of change in root number with illumination treatment : Spiraea 'Froebelii'.

	Root number		
	Illumination hours		
	High 8	High 16	Low 16
Observed	16	6	24
Expected	18.5		

$\chi^2 = 15.3$ ($p < .01$)

Table 141.

Daylength and change in mean root number : Spiraea 'Froebelii'.

	Daylength	
	8 hours	16 hours
Observed	44	30
Expected	36	36

χ^2=2.7 (N.S.)

Light intensity and change in mean root number : Spiraea 'Froebelii'.

	Intensity	
	Low	High (see text)
Observed	52	22
Expected	36	36

χ^2=12.56 ($p<.001$)

4.34 DISCUSSION

The pattern of root formation after shoot subculture on medium containing BA was similar to that for shoot initiation in that root initiation decreased with propagative generation. This indicates two possibilities :- 1) shoot condition determines potential for root formation, or 2) the same factor/s control differentiation in shoots and roots.

The first hypothesis is supported by evidence presented in Chapter 3 which showed that shoot length and leaf size are reduced with increasing number of subculture periods. Also, reduced light intensity and daylength increased shoot length and root initiation. In this case, there may be an interaction between reduced light received by the shoot and shoot elongation, and between reduced light and root initiation (as indicated in Section 4.1). However, an interaction between shoot length and root initiation cannot necessarily be inferred as increase in shoot length and root number may be separate and independent events.

On the other hand, 2iP and GA treatment in the previous generation did not promote rhizogenesis in

ninth generation shoots, although shoot elongation occurred (see Section 3.32). This indicates that shoot condition, as expressed by morphology, does not limit root initiation.

No significant decrease in root initiation with propagative generation was found in Chaenomeles. As discussed in Chapter 3, this species formed exclusively axillary shoots in culture, whereas shoots for other species used in the current experiment were adventitious in origin. This difference in shoot type may account for the observed difference in rooting potential. Sriskandarajah et al. (1982) found that progressively more apple shoots (axillary shoots) rooted with increasing number of subcultures. However, they noted that continuous illumination was essential for this effect and this was not given in my experiments. If adventitious and axillary shoots have different potential for organogenesis when cultured in similar nutrient, carbohydrate, growth regulator and environmental conditions, then the controls for such differentiation are likely to be endogenous factors.

In Spiraea, it was found that rooting potential in 2iP treated shoots did not decline as rapidly with increase in number of subcultures as in BA-treated

shoots, although a decline was still evident. This shows that decline in rooting with subculture may be regulated in part by prior cytokinin treatment. However, cytokinin does not account for the whole decline effect and therefore an interaction between a cytokinin effect and vigour of the shoots is proposed to account for the change in rooting with increasing number of subculture periods.

Reduced plant survival was evident in plants successfully rooted after many generations in culture. Culture of shoots *in vitro* leads to water vapour saturation of the atmosphere within the culture vessel. Surface wax production is inhibited under such conditions (Brainerd and Fuchigami, 1981), and retardation of stomatal development occurs (Wardle et al., 1983). This could explain the poor survival rate. The plants were less vigorous than those formed from greenhouse-rooted cuttings.

4.4 RHIZOGENESIS IN CALLUS

Section 3.4 demonstrated that shoot formation from callus is a rare occurrence in the species tested. Other workers have found that roots form from callus more often than other organs (Dodds and Roberts 1982). If roots form, the callus has not lost its capacity for differentiation. This section investigates rhizogenesis in different callus types.

4.41 ROOT FORMATION FROM CALLUS

Method

Experiment 1.

The species used in this experiment was Rhododendron concinnum. Three types of callus formed in experiments detailed in Chapter 5, were selected :- 1) lumpy friable callus, 2) glistening white flat callus, and 3) green callus (see 5.1 for details of growth regulators previously applied). IBA was incorporated in the nutrient medium at 0, 0.5, 1.0, 2.5, 5.0, 10.0, or 20.0 mg l^{-1}. Nutrient medium was either solid (gelled with agar)

or liquid (with callus supported on a filter paper bridge). Agar has been shown to adversely affect callus growth (see Section 2.23), and therefore, was eliminated in part of this experiment.

Results were recorded after 4, 8 and 12 weeks of incubation in light (16 hour photoperiod) or darkness.

Experiment 2.

The species selected for this experiment was Chaenomeles japonica because callus formation was promoted by both auxin and cytokinin. Callus derived from three sources was selected as follows :-

1) IBA callus
2) BA callus
3) 2iP callus.

IBA was incorporated in the nutrient medium at the concentrations detailed in Experiment 1 above.

Cultures were inspected for root formation after 4, 8 and 12 weeks incubation in light (16 hour photoperiod) or darkness.

Experiment 3.

Callus, initiated on medium containing IBA, of the following species was cultured on nutrient medium containing IBA at 0, 0.5, 1.0, 2.5, 5.0 or 10.0 mg l^{-1} :- Spiraea 'Froebelii', Potentilla 'Coronation Triumph, Prunus cerasifera, Arctostaphylos uva-ursi, Rhododendron 'Chikor', Rhododendron 'PJM Victor'. The nutrient medium was either stationary (gelled with agar) or liquid. Liquid media were either shaken or callus was supported on a filter paper bridge.

Root formation was recorded after 8 weeks in light (16 hour photoperiod) or darkness.

Experiment 4.

Callus of types (1) and (2), detailed in Experiment 1 above, of Rhododendron concinnum was subcultured six times to medium identical to that which had induced the initiation of the callus and in similar environmental conditions (see Section 5.1). Callus from the second,ection 5.1). Callus from the second, fourth and sixth subcultures was transferred to medium containing IBA at the concentrations specified for Experiment 1 above. Incubation was in light (16 hour photoperiod) or darkness. Cultures

were inspected for root formation every week for 12 weeks.

Results

Experiment 1.

Callus of Type 1 (lumpy friable callus) formed some roots at low IBA concentrations (0.5 and 1.0 mg l^{-1}) on gelled media in light (Table 142). Roots were not formed at lower or higher IBA concentrations and significantly more roots ($p<.01$) were formed at 1.0 than at 0.5 mg l^{-1} (Table 143). No roots formed in less than 5 weeks and most roots formed between the 9th and 12th week of culture ($p<.001$) (Table 144). Roots were very short.

In darkness, considerable callus proliferation occurred but no roots formed. Callus types 2 and 3 failed to produce any roots. No roots formed in liquid media.

Experiment 2.

No roots formed in any treatment.

Experiment 3.

All species of Rosaceae formed stubby roots in both solid and liquid stationary cultures in light (Tables 145 and 146). Media containing 2.5 mg l^{-1} or more IBA were ineffective in promoting root formation in all species tested except Arctostaphylos. Media without IBA were ineffective for rooting of all species. When using effective IBA concentrations (0.5 or 1.0 mg l^{-1}), liquid media promoted root initiation to a significantly greater extent than solid media in all species ($p<.01$) (Table 147). This was shown for both families independently when 1.0 mg l^{-1} IBA treatments were compared : Rosaceae - $p<.05$, Ericaceae - $p<.001$ (Tables 148 and 149). No difference in rooting was observed between liquid and solid media for Rosaceae at 0.5 mg l^{-1} IBA.

Roots were also formed in agitated media in Potentilla (Plate 16). No roots formed in agitated media in the other species tested.

No roots formed in darkness in any species.

Experiment 4.

No roots were formed in any treatment in either type of callus.

Table 142.

Change in mean root number derived from callus with time : Rhododendron concinnum.

IBA mg l^{-1}	Weeks			
	0	4	8	12
0	0	0	0	0
0.5	0	0	0	2.25
1.0	0	0	0.75	5.75
2.5	0	0	0	0
5.0	0	0	0	0
10.0	0	0	0	0
20.0	0	0	0	0

Table 143.

Analysis of change in root number with IBA concentration : Rhododendron concinnum.

	Root number	
	IBA mg l^{-1}	
	0.5	1.0
Observed	9	26
Expected	17.5	17.5

$\chi^2 = 8.26$ ($p < .01$)

Table 144.

Analysis of change in root number derived from callus with time : Rhododendron concinnum.

	Root number	
	Time	
	8	12 weeks
Observed	3	32
Expected	17.5	17.5

$\chi^2 = 24.03$ ($p < .001$)

Table 145.

Change in mean root number solid medium.

Species	IBA mg l^{-1}					
	0	0.5	1.0	2.5	5.0	10.0
Spiraea 'Froebelii'	0	0.75	0.75	0	0	0
Potentilla 'Coronation Triumph'	0	2.5	1.5	0	0	0
Prunus cerasifera	0	2.25	1.25	0	0	0
Arctostaphylos uva-ursi	0	0	0	0.75	0	0
Rhododendron 'P.J.M. Victor'	0	0	0	0	0	0
Rhododendron 'chikor'	0	0	0	0	0	0

Table 146.

Mean root number liquid medium.

Species	IBA mg l^{-1}					
	0	0.5	1.0	2.5	5.0	10.0
Spiraea 'Froebelii'	0	4.25	2.75	0	0	0
Potentilla 'Coronation Triumph'	0	2.5	2.0	0	0	0
Prunus cerasifera	0	0	2.5	0	0	0
Arctostaphylos uva-ursi	0	0	2.0	0	0	0
Rhododendron 'P.J.M. Victor'	0	0	0.75	0	0	0
Rhododendron 'chikor'	0	0	0.75	0	0	0

Table 147.

Analysis of difference in root number for liquid and solid media.

	Root number	
	Solid	Liquid medium
Observed	37	70
Expected	53.5	53.5

$\chi^2 = 10.2$ ($p<.01$)

Table 148.

Analysis of difference in root number for liquid and solid media : Rosaceae.

	Root number	
	Solid	Liquid medium
Observed	14	29
Expected	21.5	21.5

$\chi^2 = 5.23$ ($p<.05$)

Table 149.

Analysis of difference in root number for liquid and solid media : Ericaceae.

	Root number	
	Solid	Liquid medium
Observed	0	14
Expected	7	7

$\chi^2 = 14$ ($p<.001$)

Plate 16.

Roots formed from callus of Potentilla cultured in agitated liquid medium.

4.42 DISCUSSION

Root formation in Rhododendron concinnum occurred only on one type of callus (lumpy friable callus). This shows that 1) other types of callus may have lost their capacity for differentiation or 2) auxin sensitivity may be changed as a result of prior growth regulator treatment. Type (1) callus of all other species tested also formed roots on medium containing IBA.

It has been shown that certain types of callus differentiate more readily than others. Embryonic callus of cereals could be visually distinguished from non-embryonic callus (Nabors et al., 1983). The former consists of small isodiametric cells, whereas the latter consisted of long tubular cells (see also Section 3.42). In particular, prior treatment with 2,4-D inhibits organized development (see Section 3.42). However, it cannot be determined from the results of experiments here whether callus of types 2 and 3 has lost its capacity for differentiation irreversibly or whether differentiation could occur if other conditions were provided.

Other conditions have induced root formation in

other species, for example, culture on a medium with a high exogenous auxin / cytokinin ratio promoted rooting in callus of tobacco (Skoog, 1971) and Petunia (Durand et al., 1973); and culture on a medium with the same exogenous auxin and / or cytokinin concentration promoted root initiation in leaf callus of Lycopersicon (2 mg l^{-1} IAA + 2 mg l^{-1} kinetin) (shoots formed with 4mg l^{-1} IAA + 4 mg l^{-1} kinetin) (Padmanabhan et al., 1974). These reports show that root formation could occur under conditions not tested in the current experiment.

Roots formed when both solid and liquid media were used, thus indicating that impurities in agar (see Section 2.23) do not prevent rhizogenesis. However, root formation was greater when a liquid medium was used, suggesting that these impurities may reduce root formation.

Darkness inhibited root formation. This has also been shown by other workers (Gautheret, 1969; Letouze and Beauchesne, 1969; Lovell and Moore, 1969; Rucker and Paupardin, 1969; Weis and Jaffe, 1969). However, the results reported by these authors and the results reported here appear to conflict with the results presented in Section 4.1. In those experiments, light

was inhibitory to rooting. It is possible therefore, that darkness promotes dedifferentiation - this is supported by the fact that IBA-stimulated callus formation was inhibited by light - but that differentiation of roots is promoted by light.

After callus subculture, no roots were formed. This supports the results of other workers who noted a decline in differentiation in callus after prolonged periods in culture (Narayanaswamy, 1977).

V. DEDIFFERENTIATION

5.1 THE EFFECT OF EXOGENOUS GROWTH REGULATORS ON CALLUS FORMATION

Although callus is not necessarily an intermediate in shoot and root formation, the process of dedifferentiation must occur before shoots and roots can be initiated from stem tissue. As callus formation is essentially dedifferentiation, a study of the factors involved in its development may provide information regarding dedifferentiation. By comparing results from this section with results from Chapters 3 and 4, it should be possible to determine the controlling factors of the two separate processes of dedifferentiation and differentiation in shoot and root initiation.

Callus can be formed from some plant tissues without the addition of growth regulators, for example, immature lemon fruit (Kordan, 1959). However, most tissues require an exogenous supply of growth regulators. Yeoman and Macleod (1977) divided plant tissues into four categories with respect to their growth regulator requirements for callus formation.

These categories were 1) tissues requiring only an auxin; 2) tissues requiring only a cytokinin; 3) tissues requiring both an auxin and a cytokinin; and 4) tissues which will only respond to media containing complex natural extracts (eg coconut milk).

Experiments were conducted to determine 1) the activity of auxins and cytokinins in promotion of callus formation; 2) the most effective concentrations of these (for comparison with effective concentrations in shoot and root formation); 3) the quality of callus produced in different treatments; and 4) similarities and differences between species with respect to callus formation.

Environmental conditions are important in the control of callus formation (Murashige, 1973a). In the current experiments, the role of light and temperature were investigated in relation to exogenous growth regulator applications.

5.11 Auxins

Method

Experiment 1.

IBA at the following concentrations was incorporated in the nutrient medium :- 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}. Shoot explants of the species listed below were used.

Ericaceae:- *Arctostaphylos media*, *Arctostaphylos uva-ursi*, *Erica carnea*, *Gaultheria hispidula*, *Kalmia angustifolia* (pink form), *Rhododendron arboreum*, *R. chamae-thomsonii*, *R.* 'Chikor', *R.* 'Chinsayii', *R. dauricum*, *R. fastigiatum*, *R. forrestii*, *R. keiskei*, *R. leucaspis*, *R. lutescens*, *R.* 'P.J.M. Victor', *R. racemosum*, *R.* 'Vuyk's rosy red', *R. williamsianum*, *Vaccinium vitis-idaea*.

Rosaceae:- *Chaenomeles japonica*, *Cotoneaster dammeri*, *Crataegus brachyacantha*, *Crataegus* 'Toba', *Malus* 'Dainty', *Malus* 'Golden Hornet', *Potentilla* 'Coronation Triumph', *Potentilla* 'Sutter's Gold', *Prunus cerasifera*, *Prunus tomentosa*, *Pyracantha coccinea*, *Spiraea*

'Froebelii'.

Cultures were incubated either in continuous darkness or in light (16 hour photoperiod). Presence or absence of callus visible with the aid of a hand lens (X 10 magnification) was recorded at the end of a four week incubation period. Observations on cell aggregation, texture and colour of the callus were also noted.

Experiment 2.

A more detailed study was made of callus formation in Rhododendron concinnum. IBA, NAA or 2,4-D were incorporated in the nutrient medium at the following concentrations :- 0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 or 20 mg l^{-1}.

Diameter of callus produced at the cut end of the explants was recorded after incubation in either dark or light (16 hour photoperiod) for four weeks. Callus diameter was measured perpendicular to the stem axis.

Number of cultures in which callus formed was recorded at the end of each week for four weeks for IBA (12.5 mg l^{-1}), NAA (2.5 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}) in light and dark.

Experiment 3.

Shoot explants of Rhododendron concinnum were cultured on medium containing NAA at 2.5 mg l^{-1} for 0, 1, 4, 7, 14, 21 or 28 days, followed by culture on medium containing no auxin for the remainder of the 28 day incubation period. Incubation was in light (16 hour photoperiod) or in darkness. Number of cultures in which callus formed was recorded after 28 days.

Experiment 4.

Shoot explants of Rhododendron concinnum were incubated in darkness or in light (16 hour photoperiod) at the following temperatures:- 17, 21, 25, 29 or 33°C. NAA was incorporated in the nutrient medium at 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}. Presence or absence of callus was recorded at the end of a four-week incubation period.

Results

Experiment 1.

The number of cultures in which callus was produced after four weeks of incubation is shown in Figures 340 to359 for Ericaceae and Figures 360 to 371 for Rosaceae. Callus was formed when IBA was in the concentration range 0.5 to 20 mg l^{-1}. Concentration for maximal production of callus varied with species and cultivar. In the absence of IBA, callus was not produced in any of the species tested.

A greater number of dark-incubated cultures formed callus than light-incubated cultures in all species tested. Seven Ericaceous species and five Rosaceous species produced callus only in darkness. Plate 17 shows callus formed in light and Plate 18 shows callus formed in darkness.

Experiment 2.

Number of cultures formed on medium containing IBA, NAA or 2,4-D is shown in Figures 372 to 374). No callus formed in light on medium containing IBA, and no callus formed in the absence of exogenous auxin.

Diameter of callus produced at the cut end of the

explants is shown in Figures 375 to 377. There was no difference between callus diameter in light and dark treated cultures when incubated on medium containing 2,4-D. However, incorporation of NAA in the medium resulted in less callus in light than in darkness (Figure 383). 2,4-D treatments gave significantly more callus ($p<.001$) than NAA or IBA (Table 150; Figure 378). The order of effectiveness in callus formation and growth was (1) 2,4-D, (2) NAA, (3) IBA.

Callus was formed in most cultures between 2nd and 4th week of culture. Callus was formed earlier (weeks 1 to 3) on medium containing 2,4-D than with NAA or IBA (Table 151).

Experiment 3.

Duration of exposure to NAA necessary for callus initiation was from 4 to 7 days at 2.5 mg l NAA (Tables 152 and 153). There was no difference in exposure period required between light and dark incubated cultures.

Experiment 4.

Callus formation at different temperatures is shown in Figures 379 to 383. Callus was initiated in the greatest number of cultures at 25° C. Some callus was produced at all temperatures tested. More callus formed in darkness than in light at all temperatures tested.

Callus quality

Two types of callus were formed:- (1) lumpy friable callus, cream to brown; (2) smooth flat callus, glistening white.

Callus Type (2) was produced only a) when 2,4-D concentration was above 0.5 mg 1 or b) when the incubation temperature was 33 C. Under all other experimental conditions tested, callus was of Type (1). However, Callus Type (1) varied with auxin concentration. At higher concentrations, callus was flatter, smaller grained and more friable than at low auxin concentrations. At low auxin concentrations callus was nodular. This transition was gradual.

In addition to formation on the cut ends of explants, callus sometimes formed where leaves had been removed from the stem. Less callus was produced at

these points.

Callus originated from both the cambium and the pith and consisted mainly of parenchyma cells. Some localized centres of meristematic activity were evident.

Explants treated with 2,4-D were swollen, especially at the basal end.

Figures 340 to 359.

Number of cultures in which callus was formed in a four week incubation period on medium containing IBA : Ericaceae.

Figures 360 to 371.

Number of cultures in which callus was formed in a four week incubation period on medium containing IBA : Rosaceae.

Fig340. Arctostaphylos media.

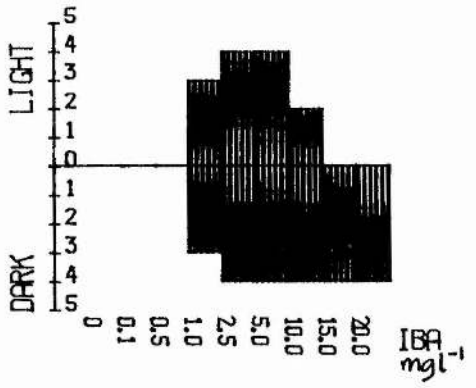


Fig341 Arctostaphylos uva-ursi.

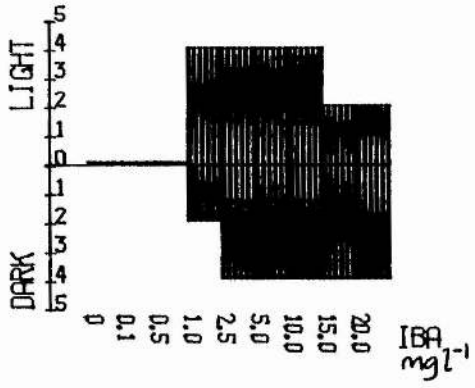


Fig 342. Erica carnea.

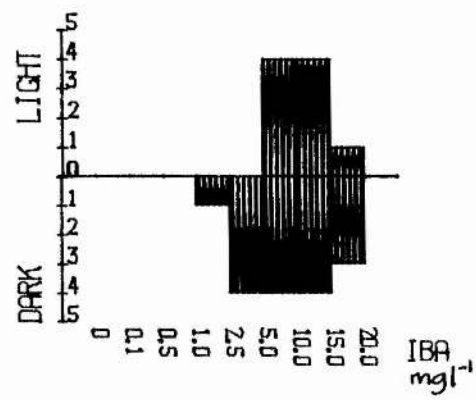


Fig343. Gaultheria hispidula.

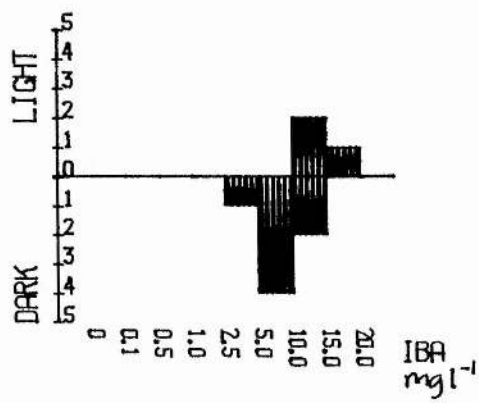


Fig 344. Kalmia angustifolia.

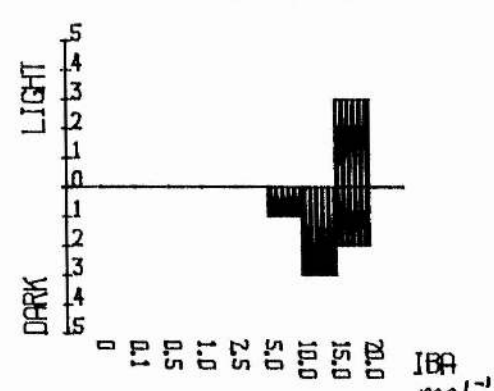


Fig 345. Rhododendron arboreum.

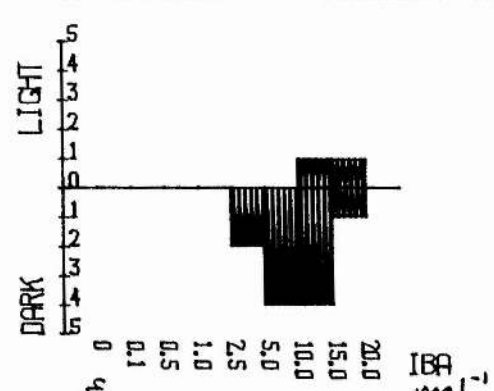


Fig 346. Rhododendron chamaethomsonii.

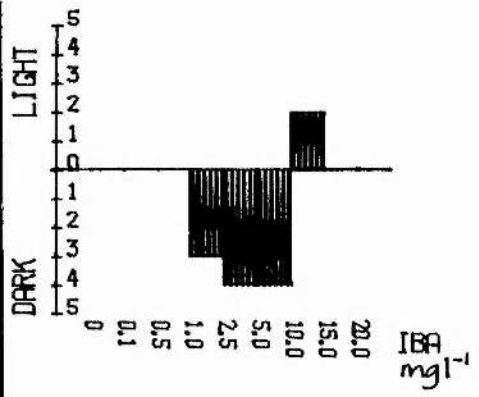


Fig 347. Rhododendron chikor.

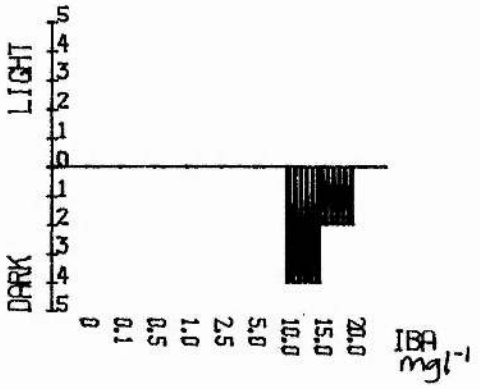


Fig 348. Rhododendron chinsayii.

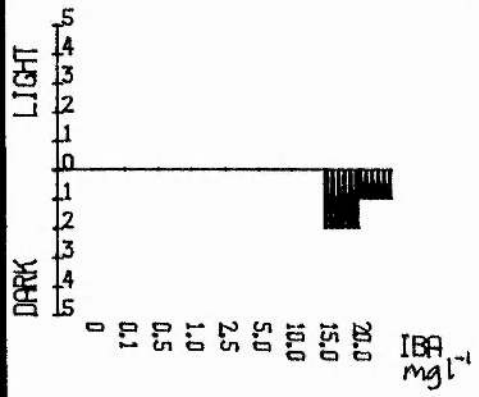


Fig 349. Rhododendron dauricum.

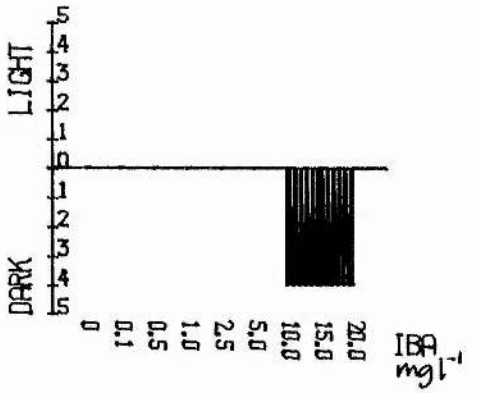


Fig 350. Rhododendron fastigiatum.

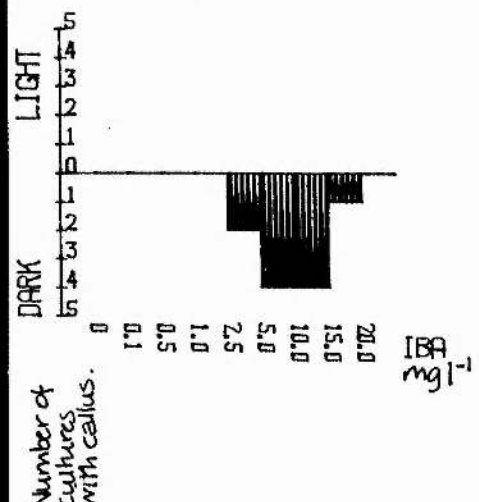


Fig 351. Rhododendron forrestii.

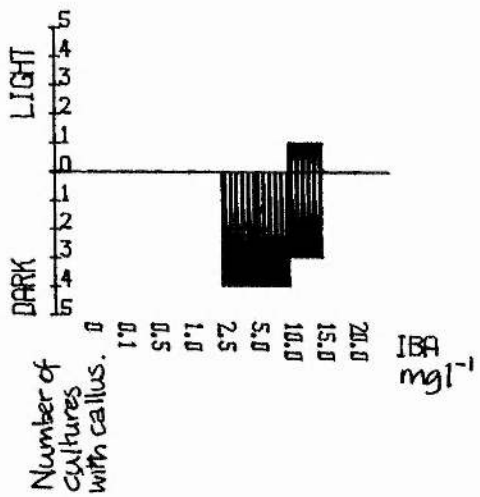


Fig 352. Rhododendron kelsket.

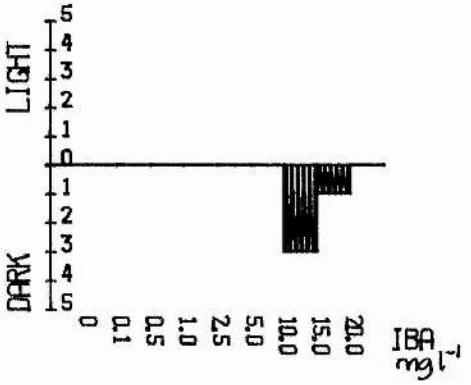


Fig 353 Rhododendron leucaspis.

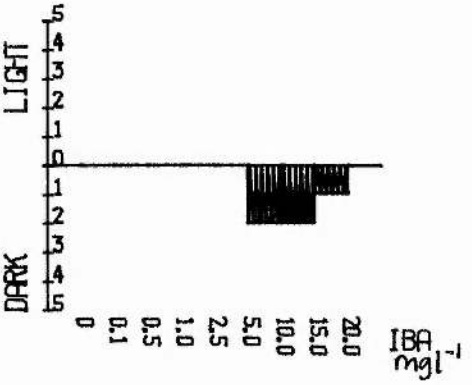


Fig 354 Rhododendron lutescens.

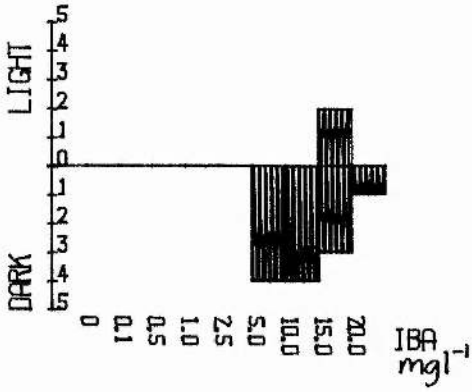


Fig 355 Rhododendron PJM Victor.

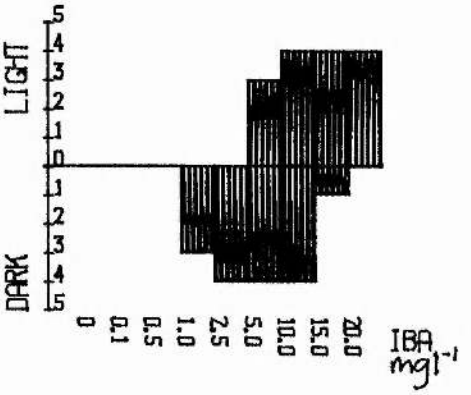


Fig 356 Rhododendron racemosum.

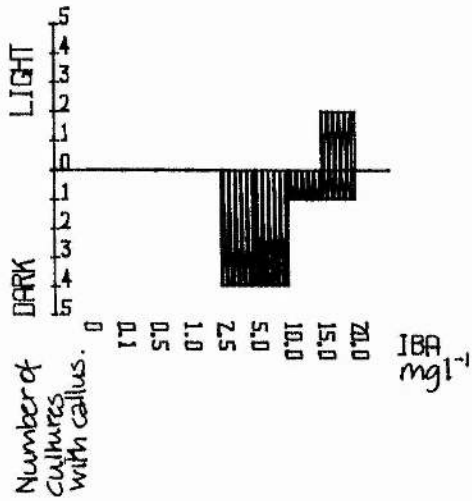


Fig 357 Rhododendron vuyks.

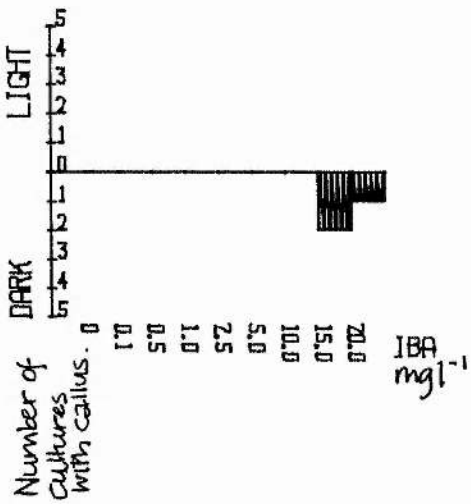


Fig 358 Rhododendron williamsianum.

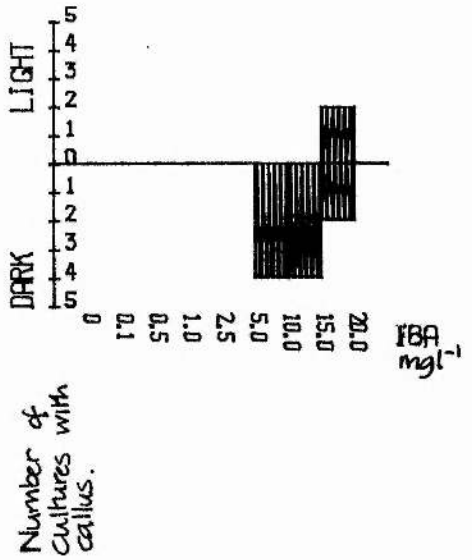


Fig 359 Vaccinium vitis-idaea.

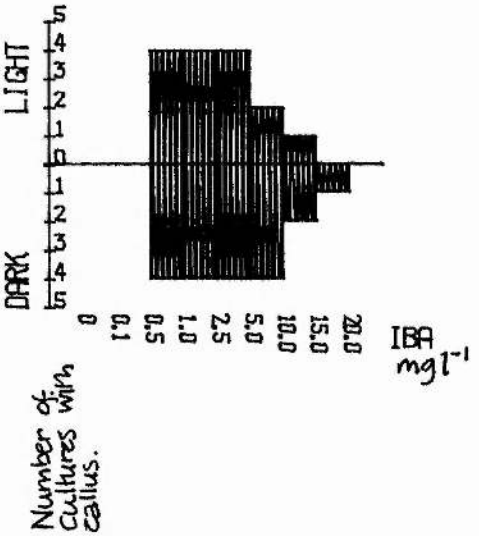


Fig 360 Chaenomeles japonica.

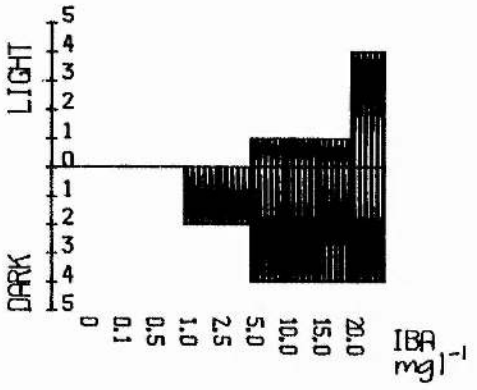


Fig 361. Cotoneaster dammeri.

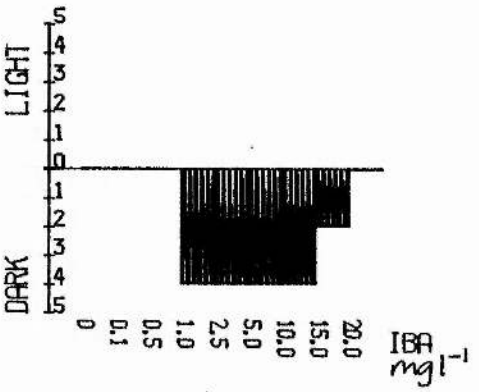


Fig 362. Crataegus brachyacantha.

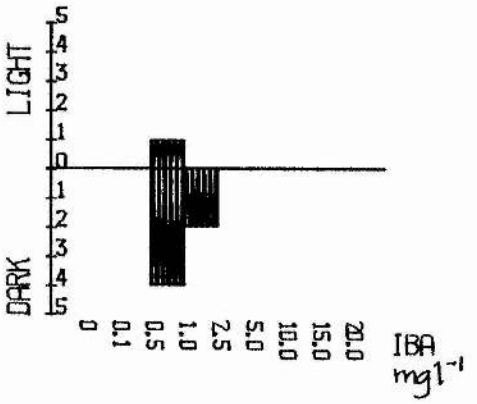


Fig 363 Crataegus Toba.

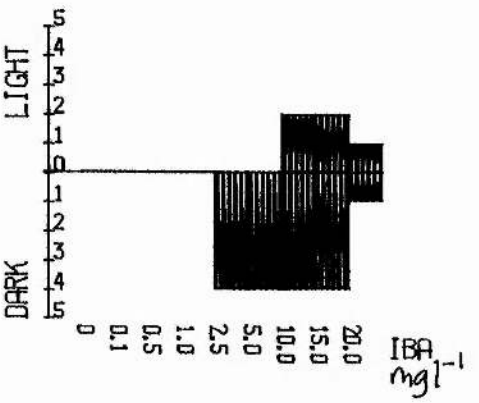


Fig 364. Malus Dainty.

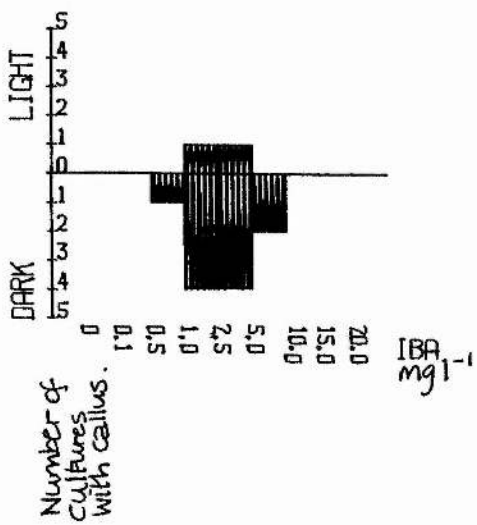


Fig 365. Malus Golden Hornet.

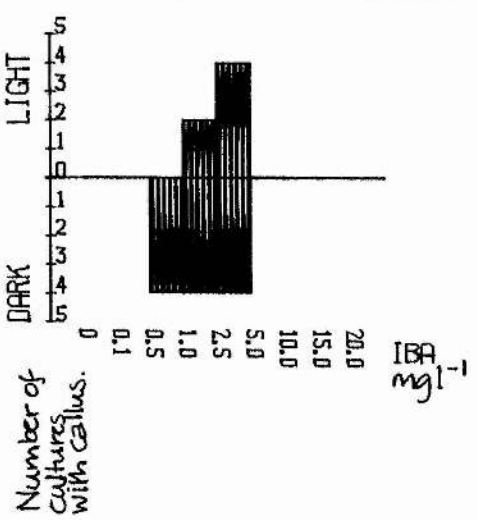


Fig 366 Potentilla Coronation Triumph.

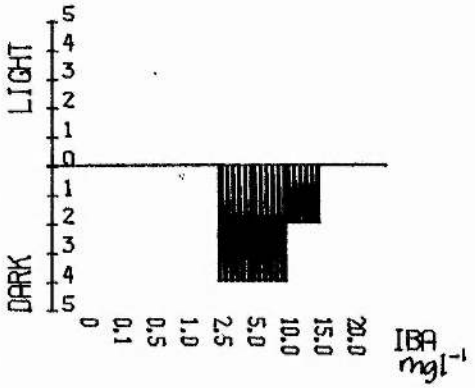


Fig 367. Potentilla Sutters Gold.

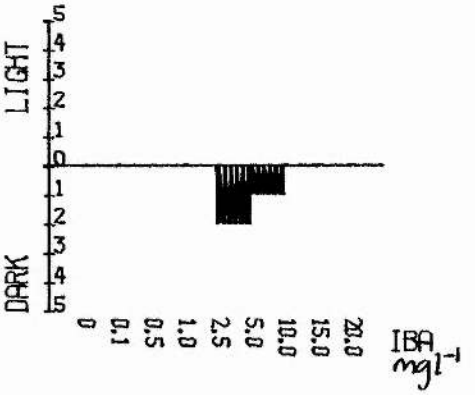


Fig 368 Prunus cerasifera.

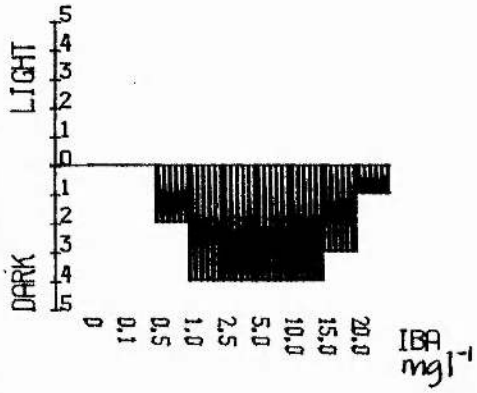


Fig 369. Prunus tomentosa.

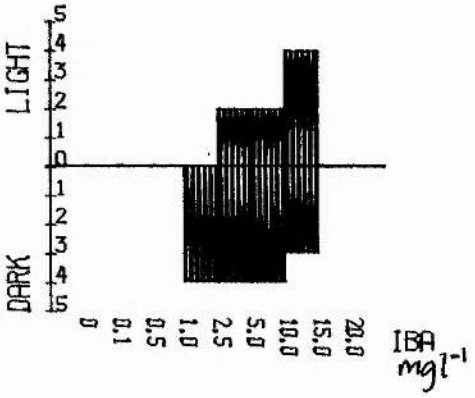


Fig 370. Pyracantha coccinea.

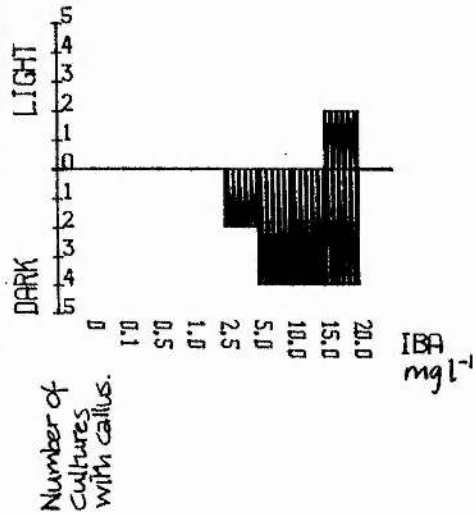
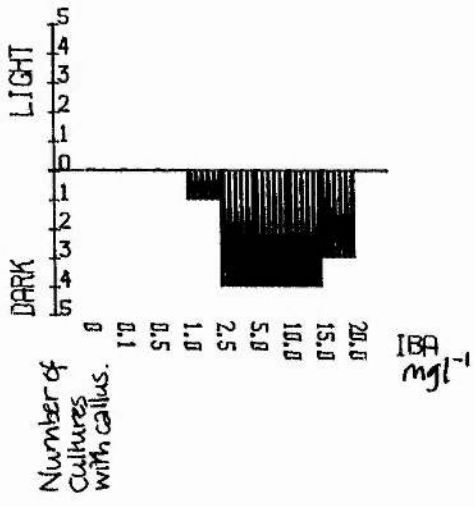


Fig 371. Spiraea Froebelii.



Figures 372 to 374.
Number of cultures in which callus formed in a four week incubation period on medium containing IBA, NAA or 2,4-D : Rhododendron concinnum

Figures 375 to 377.
Mean callus diameter at the end of a four week incubation period on medium containing IBA, NAA or 2,4-D : Rhododendron concinnum.

Figure 378.
Mean callus diameter (of cultures which formed callus) at the end of a four week incubation period on IBA, NAA or 2,4-D ; Rhododendron concinnum.

Figures 379 to 383.
Number of cultures in which callus formed in a four week incubation period at 17, 21, 25, 29 or 33 ^{0}C ; Rhododendron concinnum.

Fig 372. IBA callus

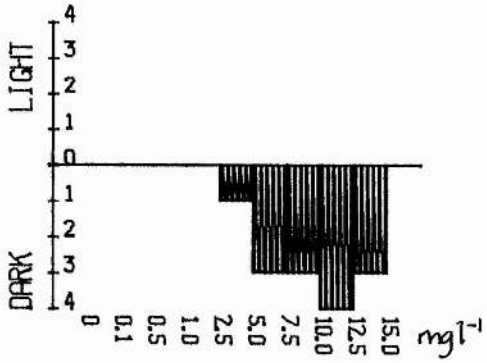


Fig 373. NAA callus

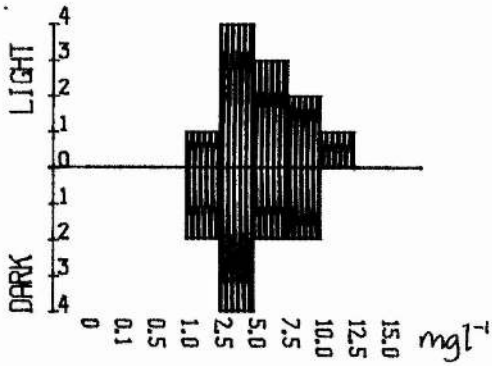
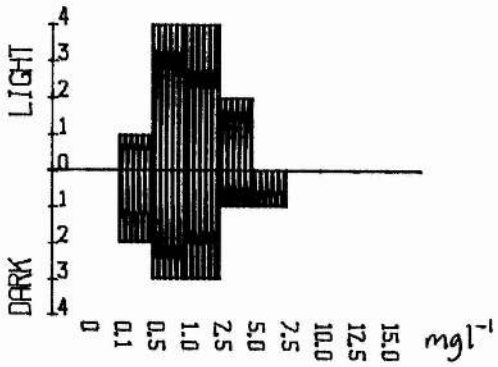


Fig 374. 2,4-D callus



Number of cultures with callus.

Fig 375. IBA mean callus diameter.

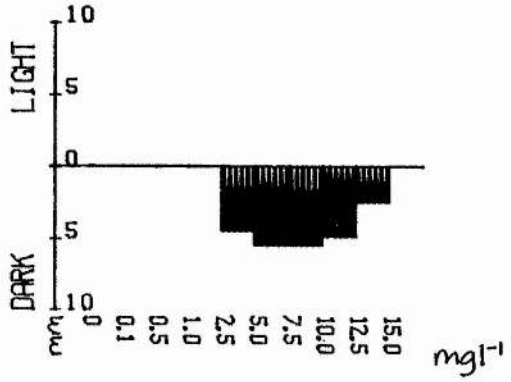


Fig 376. NAA mean callus diameter.

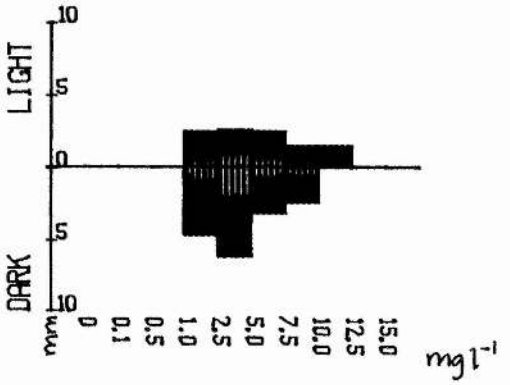


Fig 377 2,4-D mean callus diameter.

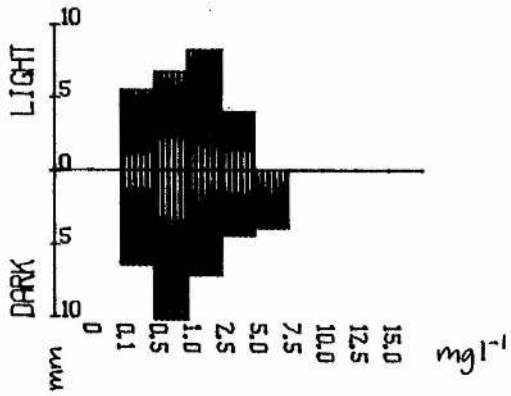


Fig 378. Mean surviving callus diameter.

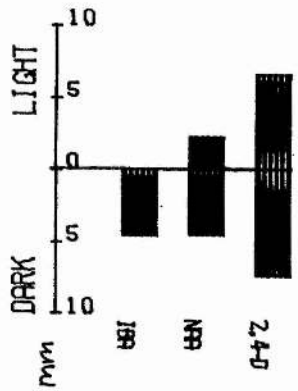


Fig 379. 17°C callus number.

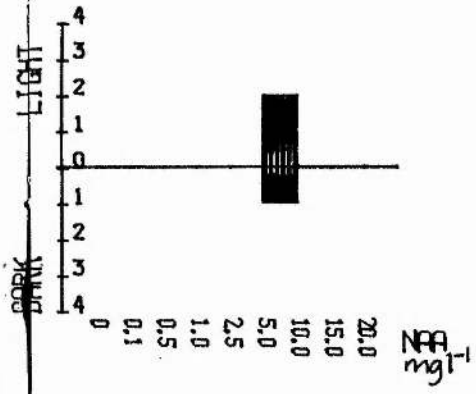


Fig 380. 21°C callus number.

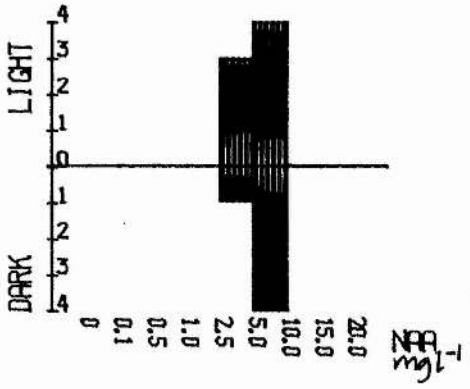


Fig 381. 25°C callus number.

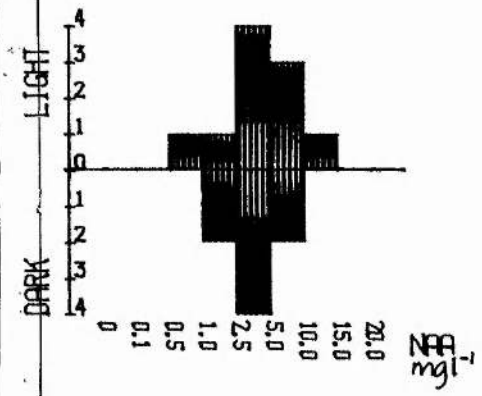


Fig 382. 29°C callus number.

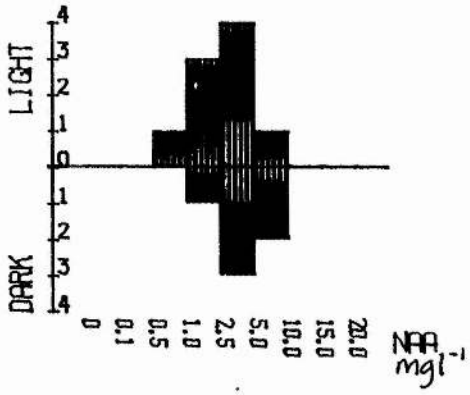
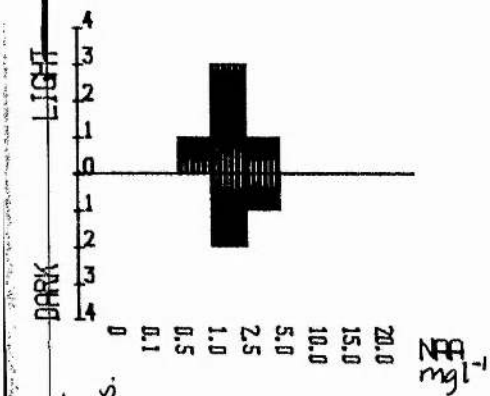


Fig 383. 33°C callus number.



Number of cultures with callus.

Number of cultures with callus.

Table 151.

Time of callus production in cultures Rhododendron concinnum.

Treatment mg l^{-1}	Number of 4 replicates with callus				
	Weeks				
	0	1	2	3	4
IBA 12.5 Dark	0	0	0	1	4
NAA 2.5 Dark	0	0	0	2	4
NAA 2.5 Light	0	0	0	1	4
2,4-D 0.5 Dark	0	1	3	4	4
2,4-D 0.5 Light	0	2	4	4	4
IBA 12.5 Light	0	0	0	0	0

Table 150.

Comparison of callus formation on media containing IBA, NAA or 2,4-D : Rhododendron concinnum.

Auxin	Mean callus diameter mm
IBA	4.06c
NAA	5.70b
2,4-D	11.24a

Means followed by different letters are significantly different ($p<.05$)

Table 152.

Duration of NAA treatment and production in cultures of Rhododendron concinnum in light.

NAA mg l^{-1}	Number of 4 replicates with callus					
	Duration of NAA treatment (days)					
	1	4	7	14	21	28
2.5	0	3	4	4	4	4
5.0	0	1	3	2	1	2
10.0	0	0	0	0	0	0

Table 153.

Duration of NAA treatment and production in cultures of Rhododendron concinnum in dark.

NAA mg l^{-1}	Number of 4 replicates with callus					
	Duration of NAA treatment (days)					
	1	4	7	14	21	28
2.5	1	4	4	4	4	4
5.0	0	2	2	3	4	3
10.0	0	1	0	0	0	0

Table 154.

Comparison of callus formation on media containing 2iP or kinetin : Rhododendron concinnum.

Cytokinin	Mean callus diameter mm
2iP	2.88a
Kinetin	2.42b

Means forllowed by different letters are significantly different ($p<.05$)

5.12 CYTOKININS

Method

A series of experiments similar to those described in 5.11 were conducted, but cytokinins were incorporated in the nutrient medium in place of auxins. The cytokinins used for Experiment (1) were BA and 2iP at the following concentrations :- 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20 mg l^{-1}. For Experiment (2), kinetin, BA and 2iP were used. 2ip was used in Experiments (3) and (4). Incubation time was four weeks.

Results

Experiment 1.

The addition of BA to the medium resulted in death of explants of *Gaultheria hispidula*, *Rhododendron* 'Chinsayii' and *R. dauricum.* BA concentrations greater than 0.1 mg l^{-1} resulted in death of explants of *Erica carnea*, *R. fastigiatum* and *R.* 'P.J.M. Victor'. This was also reported and discussed in Section 3.11. No toxic effects due to 2iP

were observed.

The number of cultures in which callus was formed is given in Figures 384 to 393 for BA treatments and Figures 394 to 405 for 2iP treatments. Callus was initiated when BA was in the concentration range 0.1 to 5.0 mg l^{-1} and when 2iP was in the concentration range 2.5 to 20.0 mg l^{-1}. Optimal concentration varied with species and cultivar.

With the exception of Arctostaphylos uva-ursi and Chaenomeles japonica, no callus was formed in darkness. A difference between species in response to the two cytokinins was noted. In Ericaceae, BA stimulated callus formation only in Arctostaphylos media, Arctostaphylos uva-ursi and Vaccinium vitis-idaea. Callus was not formed in the presence of BA in any other Ericaceous species tested. However, when supplied with 2iP, 8 of the 20 Ericaceous species formed callus.

In Rosaceae, callus was formed in the presence of BA in 8 of the 12 species tested. Cotoneaster dammeri, Malus 'Dainty', Malus 'Golden Hornet', Potentilla 'Coronation Triumph' and Potentilla 'Sutter's Gold' did not form callus in the presence of BA in either light or dark treatments. Callus was

produced in only 5 species in the presence of 2iP and, with the exception of Cotoneaster and Crataegus, less callus was formed than in the presence of BA. No Rosaceous species produced callus in darkness on 2iP-containing media. In all species, less callus was formed than in auxin treated explants.

Experiment 2.

No callus formed in darkness in any cytokinin treatment (Figures 406 and 407). No callus formed in the presence of BA. More cultures formed callus in the presence of 2iP than with kinetin.

Diameter of callus produced at the cut ends of explants is given in Figures 408 and 409. 2iP stimulated significantly ($p<.001$) more callus growth than kinetin (Table 154; Figure 410).

Visible callus formation occurred in the third week in 2iP and kinetin treatments (Table 155).

Experiment 3.

Duration of exposure period necessary for callus formation was 1 to 4 days at 15 or 20 mg l^{-1} 2iP (Table 156). A longer exposure period was necessary at lower (10 mg l^{-1}) 2iP concentration (4 to 7 days).

uva-ursi, was lumpy and friable and was similar to that produced when auxin was present and described in 5.11 as Callus Type (1).

Callus consisted primarily of parenchyma cells. Localized centres of meristematic activity were more frequent than in auxin-stimulated callus. Chloroplasts were numerous in green callus.

Figures 384 to 393.

Number of cultures in which callus formed in a four week incubation period on medium containing BA.

Figures 394 to 405.

Number of cultures in which callus formed in a four week incubation period on medium containing 2iP.

Fig 384. Arctostaphylos media.

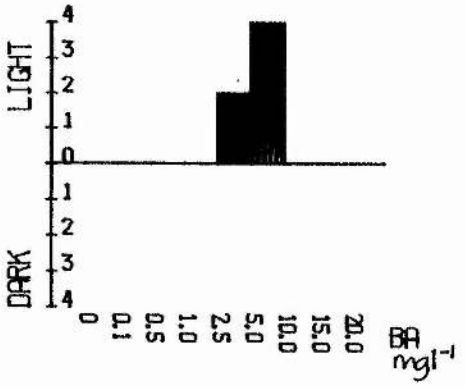


Fig 385 Arctostaphylos uva-ursi.

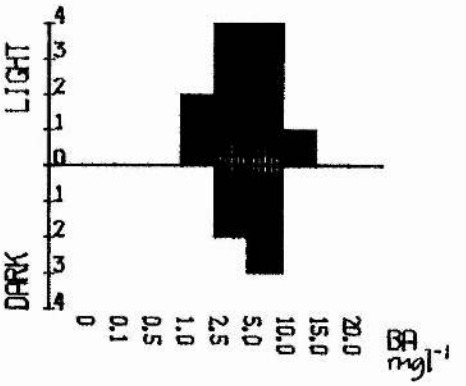


Fig 386. Vaccinium vitis-idaea.

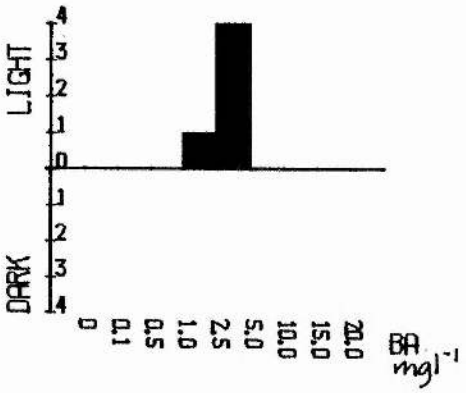


Fig 387. Chaenomeles japonica.

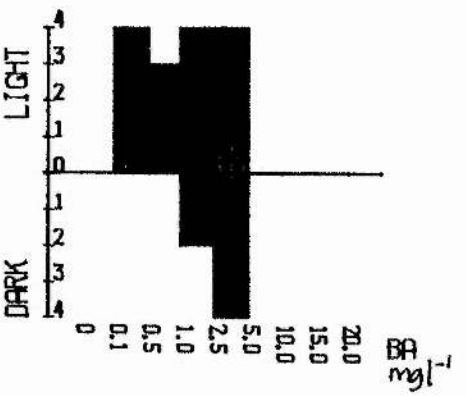


Fig 388. Crataegus brachyacantha.

Fig 389. Crataegus Toba.

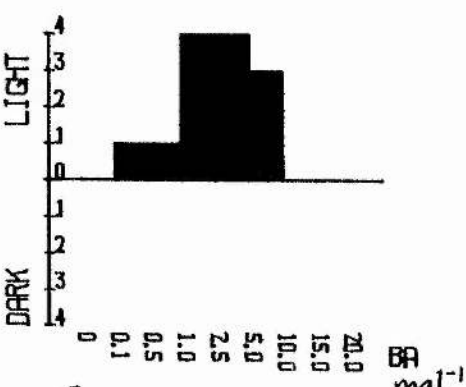


Fig 390. Prunus cerasifera.

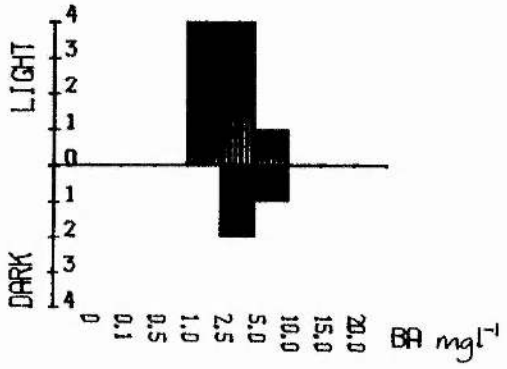


Fig 391. Prunus tomentosa.

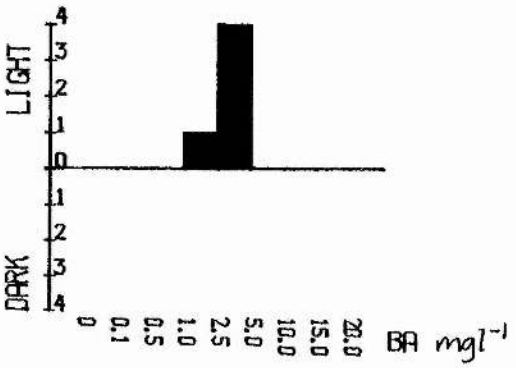


Fig 392. Pyracantha coccinea.

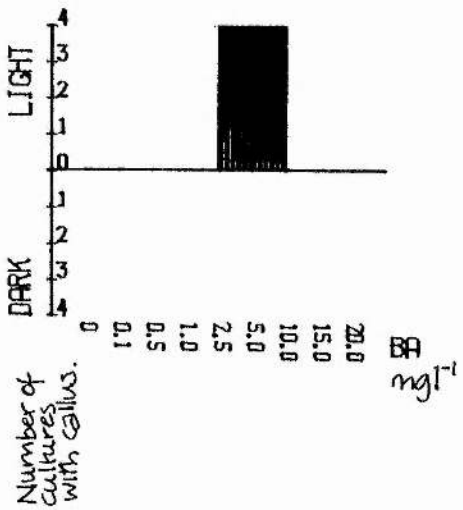


Fig 393 Spiraea Froebelii.

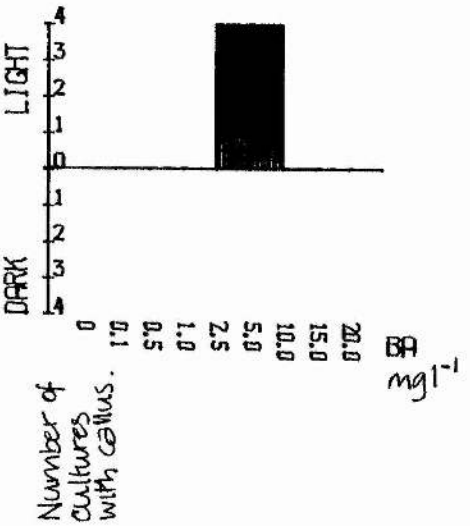


Fig 394. Arctostaphylos media.

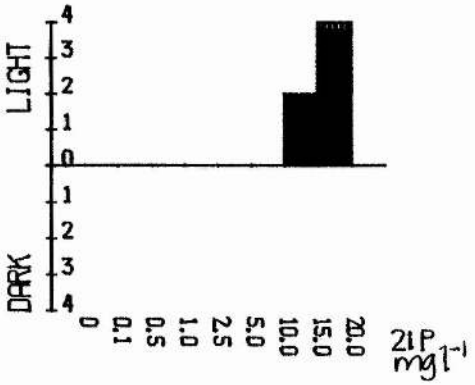


Fig 395. Arctostaphylos uva-ursi.

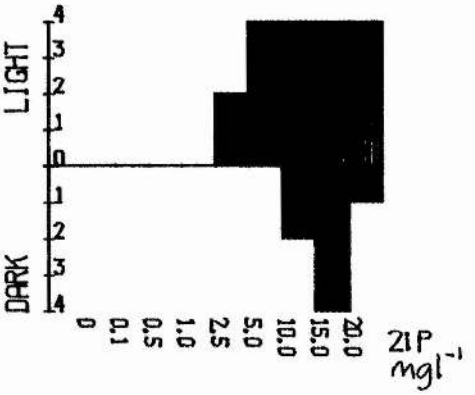


Fig 396. Erica carnea.

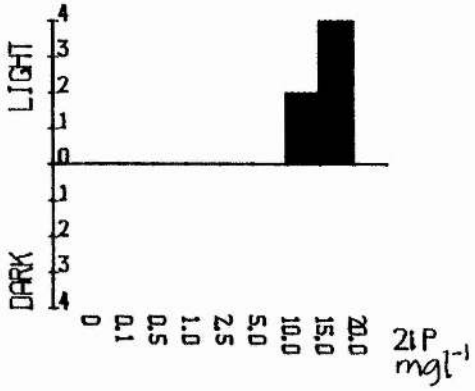


Fig 397. Rhododendron dauricum.

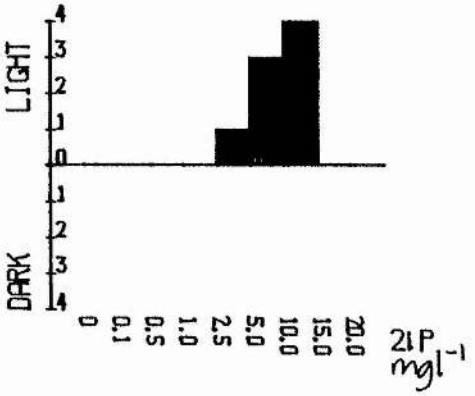


Fig 398 Rhododendron PJM' Victor.

Fig 399. Rhododendron vuyks.

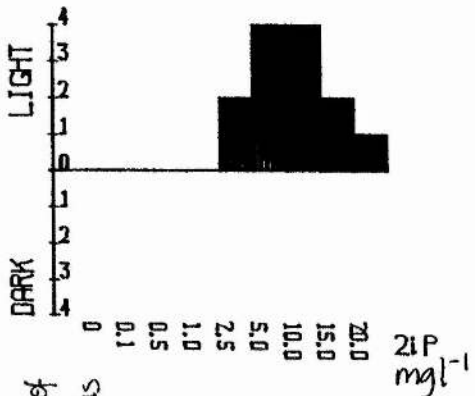


Fig 400 Vaccinium vitis-idaea.

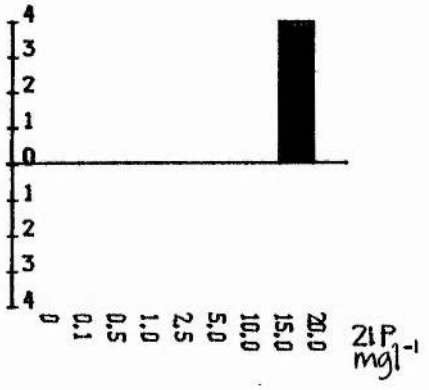


Fig 401. Chaenomeles japonica.

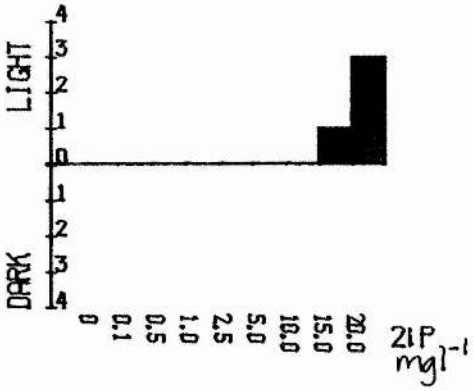


Fig 402. Cotoneaster dammeri.

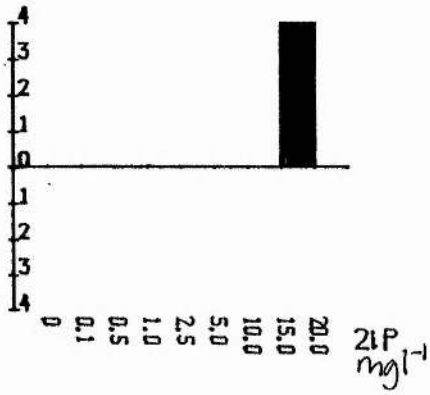


Fig 403. Crataegus brachyacantha.

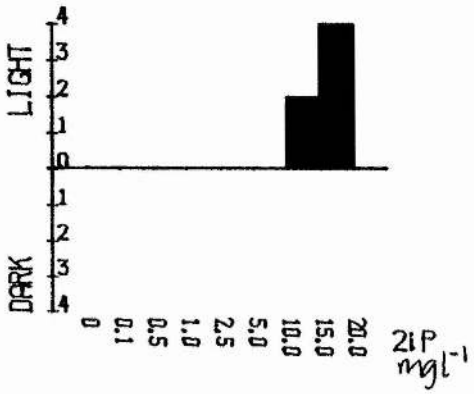


Fig 404. Crataegus Toba.

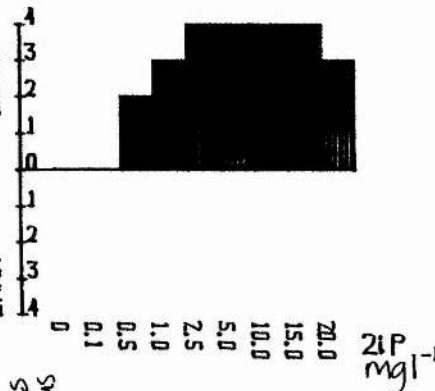
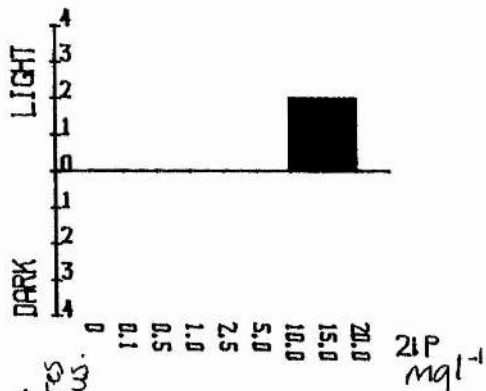


Fig 405. Prunus tomentosa.



Figures 406 and 407.
Number of cultures in which callus formed in a four week incubation period on medium containing kinetin or 2iP : Rhododendron concinnum.

Figures 408 and 409.
Mean callus diameter after a four week incubation period on medium containing kinetin or 2iP : Rhododendron concinnum.

Figure 410.
Mean callus diameter (of cultures in which callus formed) after incubation for four weeks on medium containing kinetin or 2iP : Rhododendron concinnum.

Figures 411 to 413.
Number of cultures forming callus in a four week incubation period at 21, 25 or 29 °C (no callus formed at 17 or 33°C) : Rhododendron concinnum.

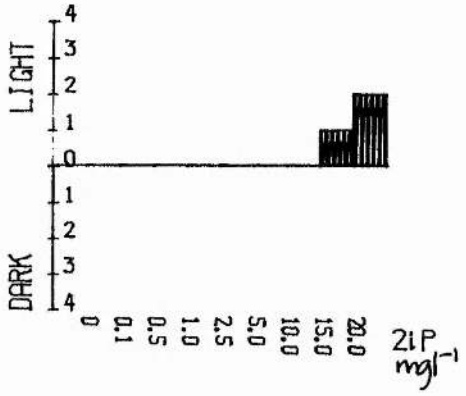
Fig 406. Kinetin.
Number of cultures with callus
LIGHT
DARK
4
3
2
1
0
1
2
3
4
0
0.1
0.5
1.0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
CK mgl⁻¹

Fig 407. 2iP.
Number of cultures with callus
LIGHT
DARK
4
3
2
1
0
1
2
3
4
0
0.1
0.5
1.0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
CK mgl⁻¹

Fig 408 Kinetin.
Callus diameter (mm)
LIGHT
DARK
10
5
0
5
10
0
0.1
0.5
1.0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
CK mgl⁻¹

Fig 409 2iP.
Callus diameter (mm)
LIGHT
DARK
10
5
0
5
10
0
0.1
0.5
1.0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
CK mgl⁻¹

Fig 411. 21°C.



Number of cultures with callus

Fig 412. 25°C.

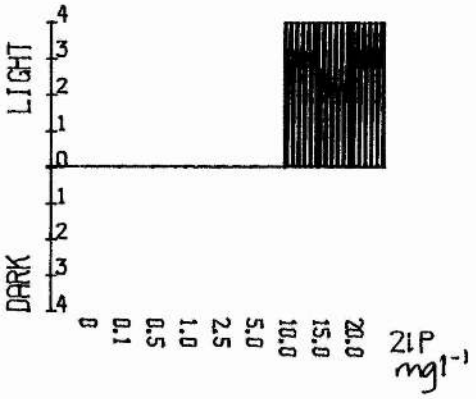


Fig 413. 29°C.

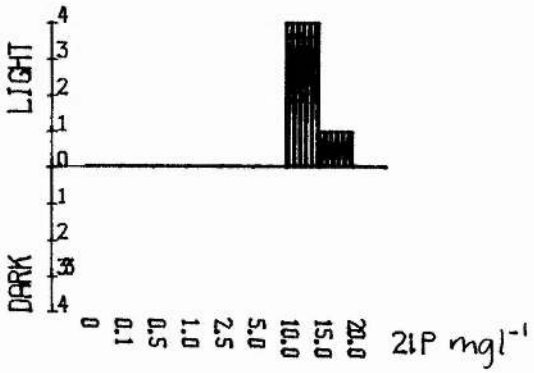


Fig 410.

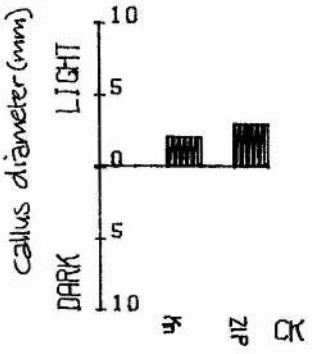


Table 155.

Number of cultures (of 4) in which callus formed with time in Rhododendron concinnum.

	Treatment			
mg l^{-1}	Number of weeks in culture			
	1	2	3	4
2iP	0	0	4	4
Kinetin	0	0	3	3

Table 156.

Duration of exposure to 2iP treatment and callus formation in Rhododendron concinnum in light (16 hour day).

	Number of cultures (of 4) with callus					
2iP mg l^{-1}	Duration of 2iP treatment (days)					
	1	4	7	14	21	28
10.0	0	2	4	4	4	4
15.0	0	4	4	4	4	3
20.0	1	4	3	4	4	4

5.13 AUXIN AND CYTOKININ INTERACTION

Method

Experiment 1.

2iP, BA or kinetin and IBA, NAA or 2,4-D at the following concentrations were incorporated in the nutrient medium :- 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 mg l^{-1} . The species tested was Rhododendron concinnum. Cultures were incubated either in darkness or in light (16 hour photoperiod). Diameter of callus produced on the cut ends of the explants visible with the aid of a hand lens (X 10 magnification) was recorded at the end of a four week incubation period in darkness or in light (16 hour photoperiod).

Experiment 2.

Auxin and cytokinin applied together may be utilized sequentially or simultaneously. This may be important in elucidating the stages involved in dedifferentiation. Experiments 2 and 3 were conducted to investigate the possibility of sequential auxin and

cytokinin action in callus formation.

Shoot explants of Rhododendron concinnum were treated with (1) a NAA quick dip (0.5% NAA) for five seconds or (2) no NAA, prior to incubation on nutrient medium supplemented with 2iP or NAA at 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}.

Callus diameter was recorded after illuminated or dark incubation after one, two, three and four weeks.

Experiment 3.

Explants were 1. pretreated with NAA as described for Experiment 2 above or 2. not pre-treated with NAA or 2ip. In addition they were given either a dark or a light (16 hour photoperiod) pretreatment for one week prior to a further three week incubation in either light or dark. Callus diameter was recorded at the end of this period.

Results

Experiment 1.

Results are given in Tables 157 to 174. Incorporation in the medium of auxin and cytokinin simultaneously enhanced the promotive effect of cytokinin or auxin and more callus growth resulted.

Analysis of variance demonstrated a significant effect on callus formation due to auxin concentration ($p<.001$), cytokinin concentration ($p<.001$) and a significant interaction between auxin and cytokinin. This was shown for all auxin and cytokinin combinations. However, a significant effect due to light/dark was only demonstrated for the following combinations :- BA+IBA, BA+NAA, 2iP+NAA, Kinetin+2,4-D, 2iP+2,4-D. There was no significant difference between light and dark treatments for the following :- Kinetin+IBA, 2iP+IBA, Kinetin+NAA, BA+2,4-D.

Experiment 2.

IBA applied as a quick dip prior to incubation did not affect the quantity of callus produced over controls (Table 175 and 176).

An increase in callus formation occurred when 2iP was given as a quick dip prior to incubation on auxin-containing medium (Table 177). Callus diameter was significantly greater ($p<.001$) than that produced when 2iP and NAA were supplied together in the nutrient medium (Table 178).

Tables 179 and 180 give callus diameter after 1, 2, 3 and 4 weeks of culture. An analysis of variance showed a significant effect ($p<.001$) due to duration of

culture, 2iP concentration and light / dark (Table 181) and, in a separate analysis, a significant effect ($p<.001$) due to duration of culture, NAA concentration, and light / dark (Table 182). Callus formation occurred more rapidly when NAA or 2iP was given as a pretreatment than when no pretreatment was given (compare data in sections 5.1 and 5.2).

Experiment 3.

Callus formation after NAA or 2iP and dark and light pretreatments is given in Tables 183 and 184. An analysis of variance showed that significantly more callus ($p<.001$) was formed in light pretreated cultures (Tables 185 and 186). A significant effect ($p<.001$) was also shown due to 2iP and NAA concentration and there was a significant interaction ($p<.001$) light / dark pretreatment and 2iP and NAA concentration.

Callus formation when cultures were dark-pretreated, was similar to that formed in continuous darkness when NAA was provided. When cultures were light incubated with NAA, no callus formation occurred until cultures were placed in darkness. Light pretreatment delayed callus development. However, more callus formation occurred in cultures which had been light pre-treated.

When 2iP was supplied, it was found that light was essential for initiation of callus formation, but darkness did not slow callus formation once the process had started.

Tables 187 and 188 give callus formation after dark and light pre-treatment but with no 2iP or NAA pre-treatment. On medium containing 2iP, an analysis of variance (Table 189) showed a significant effect ($p<.001$) on callus formation due to light/dark pre-treatment, 2iP concentration, and a significant interaction between these ($p<.001$). More callus formed in light pre-treated cultures. On NAA medium, also, more callus formed in light pre-treated cultures. An analysis of variance (Table 190) showed a significant effect due to light/dark ($p<.05$), a significant effect due to NAA concentration ($p<.001$) and a significant interaction between these ($p<.01$).

Callus quality

On all media containing auxin, callus was cream to brown in colour and was similar in appearance regardless of the presence of cytokinin.

Table 157.

Mean callus diameter (mm) after 4 weeks incubation on medium containing kinetin and IBA : Rhododendron concinnum.

mg l^{-1}	Kinetin	IBA
0	0.563	0.132
0.1	0.806	0.333
0.5	0.479	0.514
1.0	1.264	0.500
2.5	0.778	0.667
5.0	0.583	1.153
10.0	0.431	1.549
15.0	0.076	0.132
20.0	0	0
L.S.D.	0.323	0.323

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
0.494		0.613
L.S.D.	N.S.	

Table 158.

Analysis of variance for callus diameter after kinetin and BA treatment in light (16 hours) or darkness : Rhodoendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
kinetin conc.	84.535	8	10.567	11.640	<.001
IBA conc.	149.535	8	18.692	20.590	<.001
light	2.287	1	2.287	2.520	N.S.
kinetin * IBA	381.188	64	5.956	6.561	<.001
kinetin * light	128.431	8	16.054	17.685	<.001
IBA * light	210.473	8	26.309	28.981	0.001
kinetin * IBA * light	349.277	64	5.457	2.211	<.01
Error	441.188	486	0.908		
Total	1746.913	647			

Table 159.

Mean callus diameter (mm) after 4 weeks incubation on medium containing BA and IBA : Rhododendron concinnum.

mg l^{-1}	BA	IBA
0	0.563	0
0.1	0.611	0
0.5	0.958	0.132
1.0	1.0	0.160
2.5	1.160	1.361
5.0	1.521	2.389
10.0	0.431	1.896
15.0	0	0.285
20.0	0	0.0208
L.S.D.	0.270	0.270

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
0.0787		1.309
L.S.D.	0.127	

Table 160.

Analysis of variance for callus diameter after BA and IBA treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
BA conc.	152.704	8	19.088	30.261	<.001
IBA conc.	500.211	8	62.526	99.124	<.001
light	245.065	1	245.065	388.507	<.001
BA * IBA	307.934	64	4.81110	7.628	<.001
BA * light	98.612	8	12.326	19.541	<.001
IBA * light	386.535	8	48.317	76.598	0.001
BA * IBA * light	238.319	64	3.724	2.443	<.001
Error	306.563	486	0.631		
Total	2235.944	647			

Table 161.

Mean callus diameter (mm) after 4 weeks incubation on medium containing 2iP and IBA : Rhododendron concinnum.

mg l^{-1}	2iP	IBA
0	0.431	0.354
0.1	0.375	0.569
0.5	0.299	0.764
1.0	0.313	1.208
2.5	0.701	1.229
5.0	1.208	1.389
10.0	1.882	1.215
15.0	1.056	0.0903
20.0	0.556	0
L.S.D.	0.191	0.191

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
0.718		0.798
L.S.D.	0.090	

Table 162.

Analysis of variance for callus diameter after 2iP and IBA treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP conc.	162.878	8	20.360	63.889	<.001
IBA conc.	162.073	8	20.259	63.573	<.001
light	1.043	1	1.043	3.274	N.S.
2iP * IBA	348.511	64	5.445	17.088	<.001
2iP * light	146.735	8	18.342	57.557	<.001
IBA * light	319.929	8	39.991	125.493	0.001
2iP * IBA * light	206.918	64	3.233	7.195	<.001
Error	154.875	486	0.319		
Total	1502.961	647			

Table 163.

Mean callus diameter (mm) after 4 weeks incubation on medium containing kinetin and NAA : Rhododendron concinnum.

mg l^{-1}	kinetin	NAA
0	0.938	0.132
0.1	1.396	0.410
0.5	1.938	0.792
1.0	1.778	1.563
2.5	1.153	2.618
5.0	0.632	2.049
10.0	0.583	0.979
15.0	0.028	0.028
20.0	0.125	0
L.S.D.	0.339	0.339

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
0.907		0.997
L.S.D.	0.159	

Table 164.

Analysis of variance for callus diameter after kinetin and NAA treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
kinetin conc.	264.031	8	33.004	33.183	<.001
NAA conc.	511.524	8	63.940	64.288	<.001
light	1.298	1	1.298	1.305	N.S.
kinetin * NAA	833.900	64	13.030	13.100	<.001
kinetin * light	90.091	8	11.261	11.323	<.001
NAA * light	77.876	8	9.734	9.787	0.001
kinetin * NAA * light	213.423	64	3.335	1.415	<.05
Error	483.375	486	0.995		
Total	2475.517	647			

Table 165.

Mean callus diameter (mm) after 4 weeks incubation on medium containing BA and NAA : Rhododendron concinnum.

mg l^{-1}	BA	NAA
0	0.903	0
0.1	1.556	0
0.5	1.549	0.090
1.0	0.431	0.667
2.5	0.111	1.618
5.0	0	1.479
10.0	0	0.653
15.0	0	0.042
20.0	0	0
L.S.D.	0.195	0.195

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
0.505		0.506
L.S.D.	N.S.	

Table 166.

Analysis of variance for callus diameter after BA and NAA treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
BA conc.	254.290	8	31.786	95.691	<.001
NAA conc.	243.908	8	30.489	91.784	<.001
light	3.858e-04	1	3.858e-04	1.162e-03	N.S.
BA * NAA	491.627	64	7.682	23.125	<.001
BA * light	15.809	8	1.976	5.949	<.001
NAA * light	2.107	8	0.263	0.793	N.S.
BA * NAA * light	93.052	64	1.454	0.744	N.S.
Error	161.438	486	0.332		
Total	1262.231	647			

Table 167.

Mean callus diameter (mm) after 4 weeks incubation on medium containing 2iP and NAA : Rhododendron concinnum.

mg l^{-1}	2iP	NAA
0	0.875	0
0.1	0.826	0
0.5	0.771	0.090
1.0	1.056	0.667
2.5	1.326	1.618
5.0	1.243	1.479
10.0	2.090	0.653
15.0	1.236	0.042
20.0	0.639	0
L.S.D.	0.229	0.229

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
1.557		0.679
L.S.D.	0.108	

Table 168.

Analysis of variance for callus diameter after 2iP and NAA treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP conc.	109.181	8	13.648	30.038	<.001
NAA conc.	1039.306	8	129.913	285.934	<.001
light	124.908	1	124.908	274.917	<.001
2iP * NAA	367.576	64	5.743	12.641	<.001
2iP * light	296.748	8	37.094	81.641	<.001
NAA * light	62.790	8	7.849	17.274	<.001
2iP * NAA * light	385.397	64	6.022	10.205	<.001
Error	220.813	486	0.454		
Total	2606.719	647			

Table 169.

Mean callus diameter (mm) after 4 weeks incubation on medium containing kinetin and 2,4-D : Rhododendron concinnum.

mg l^{-1}	kinetin	2,4-D
0	1.951	0.132
0.1	1.924	1.160
0.5	2.083	4.271
1.0	2.083	4.542
2.5	1.944	1.299
5.0	1.396	0.201
10.0	0.146	0
15.0	0.076	0
20.0	0	0
L.S.D.	0.345	0.345

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
1.847		0.731
L.S.D.	0.162	

Table 170.

Analysis of variance for callus diameter after kinetin and 2,4-D treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
kinetin conc.	502.792	8	62.849	60.778	<.001
2,4-D conc.	1943.583	8	242.948	234.941	<.001
light	201.670	1	201.670	195.024	<.001
kinetin * 2,4-D	1144.278	64	17.879	17.290	<.001
kinetin * light	284.764	8	35.595	34.422	<.001
2,4-D * light	289.375	8	36.172	34.980	<.001
kinetin * 2,4-D * light	570.472	64	8.914	4.303	<.001
Error	502.563	486	1.034		
Total	5439.497	647			

Table 171.

Mean callus diameter (mm) after 4 weeks incubation on medium containing BA and 2,4-D : Rhododendron concinnum.

mg l^{-1}	BA	2,4-D
0	1.965	0
0.1	2.382	1.278
0.5	2.597	4.340
1.0	3.778	4.688
2.5	1.826	2.306
5.0	0.500	0.507
10.0	0	0.028
15.0	0	0
20.0	0.097	0
L.S.D.	0.578	0.578

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
1.548		1.373
L.S.D.	0.272	

Table 172.

Analysis of variance for callus diameter after BA and 2,4-D treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
BA conc.	1076.173	8	134.522	46.501	<.001
2,4-D conc.	2074.701	8	259.338	89.647	<.001
light	4.926	1	4.926	1.703	N.S.
BA * 2,4-D	1690.465	64	26.414	9.131	<.001
BA * light	54.515	8	6.814	2.356	<.05
2,4-D * light	16.723	8	2.090	0.723	N.S.
BA * 2,4-D * light	295.805	64	4.622	0.294	N.S.
Error	1405.938	486	2.893		
Total	6619.247	647			

Table 173.

Mean callus diameter (mm) after 4 weeks incubation on medium containing 2iP and 2,4-D : Rhododendron concinnum.

mg l^{-1}	2iP	2,4-D
0	1.861	0.431
0.1	2.035	2.083
0.5	2.333	7.528
1.0	2.979	6.049
2.5	3.590	2.840
5.0	2.778	1.174
10.0	2.132	0
15.0	1.355	0
20.0	1.042	0
L.S.D.	0.444	0.444

Mean callus diameter (mm) after 4 weeks incubation in light or darkness : Rhododendron concinnum.

Light (16 hour day)		Darkness
3.299		1.168
L.S.D.	0.209	

Table 174.

Analysis of variance for callus diameter after 2iP and 2,4-D treatment in light (16 hours) or darkness : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
2iP conc.	366.139	8	45.767	26.748	<.001
2,4-D conc.	4486.660	8	560.832	327.774	<.001
light	735.787	1	735.787	430.025	<.001
2iP * 2,4-D	1474.063	64	23.032	13.461	<.001
2iP * light	204.160	8	25.520	14.915	<.001
2,4-D * light	563.945	8	70.493	41.199	<.001
2iP * 2,4-D * light	1615.013	64	25.235	1.864	<.05
Error	831.563	486	1.711		
Total	10277.330	647			

Table 175.

Mean callus diameter after NAA pretreatment and incubation for 4 weeks on medium containing 2iP : Rhododendron concinnum.

2iP	Callus diameter (mm)			
mg l^{-1}	control (-auxin)		+NAA	
	dark	light	dark	light
0	0	0	0	0
0.1	0	0	0	0
0.5	0	0	0	0
1.0	0	0	0	0
2.5	0	0	0	0
5.0	0	0	0	0
10.0	0	4.13	1.00	4.50
15.0	0	2.00	1.25	2.75
20.0	0	1.75	0.88	2.00
Mean	0c	0.88a	0.35b	1.03a

Means followed by different letters are significantly different ($p<.05$)

Table 176.

Analysis of variance for data given in Table 175 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
auxin	4.786	3	1.595	58.152	<.001
2iP conc	21.465	8	2.683	97.808	<.001
Interaction	10.547	24	0.439	16.019	<.001
Error	2.963	108	0.0274		
Total	39.761	143			

a loge (y+1) transformation was used

Table 177.

Mean callus diameter after 2iP pretreatment and incubation for 4 weeks on medium containing NAA : Rhododendron concinnum.

NAA	Callus diameter (mm)			
mg l^{-1}	control (-CK)		+CK	
	dark	light	dark	light
0	0	0	0	0
0.1	0	0	0	0
0.5	0	0	5.63	7.88
1.0	3.50	1.25	6.63	9.75
2.5	6.38	2.88	11.25	12.38
5.0	0.75	2.38	7.88	8.63
10.0	0	0.88	5.50	0
15.0	0	0	0	0
20.0	0	0	0	0
Mean	1.18b	0.82b	4.10a	4.29a

Means followed by different letters are significantly different ($p<.05$)

Table 178.

Analysis of variance for data given in Table 177 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
cytokinin	18.062	3	6.021	76.767	<.001
NAA conc	86.023	8	10.753	137.104	<.001
Interaction	29.265	24	1.219	15.548	<.001
Error	8.470	108	0.0784		
Total	141.821	143			

a loge (y+1) transformation was used.

Table 179. Mean callus diameter (mm) after 1, 2, 3 and 4 weeks incubation on medium containing 2iP, NAA pretreatment : Rhododendron concinnum.

I. WEEK	diameter mm
1	0.292
2	0.535
3	0.632
4	0.688
L.S.D.	0.152
II. mg l^{-1} 2iP	diameter mm
0	0
0.1	0
0.5	0
1.0	0
2.5	0
5.0	0
10.0	1.84
15.0	1.67
20.0	1.31
L.S.D.	0.228
III. daylength	callus diameter
light	0.813
dark	0.260
L.S.D.	0.107

Table 180. Mean callus diameter (mm) after 1, 2, 3 and 4 weeks incubation on medium containing NAA, 2iP pretreatment : Rhododendron concinnum.

I. WEEK	diameter mm
1	0.944
2	1.951
3	3.424
4	4.194
L.S.D.	0.270
II. mg l NAA	diameter mm
0	0
0.1	0
0.5	4.609
1.0	5.703
2.5	6.734
5.0	5.016
10.0	1.594
15.0	0
20.0	0
L.S.D.	0.405
III. daylength	callus diameter
light	2.715
dark	2.542
L.S.D.	N.S.

Table 181.

Analysis of variance for data in table 179 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
weeks	6.614	3	2.205	10.963	<.001
2iP conc.	170.469	8	21.309	105.960	<.001
light	21.945	1	21.945	109.127	<.001
weeks * 2iP	21.378	24	0.891	4.429	<.001
weeks * light	0.787	3	0.262	1.305	N.S.
2iP * light	51.312	8	6.414	31.895	<.001
weeks * 2iP * light	5.924	24	0.2468	0.1361	N.S.
Error	43.438	216	0.2011		
Total	321.867	287			

Table 182.

Analysis of variance for data in table 180 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
weeks	459.281	3	153.094	242.258	<.001
NAA conc.	2068.497	8	258.562	409.153	<.001
light	2.170	1	2.170	3.43427	N.S.
weeks * NAA	513.281	24	21.387	33.843	<.001
weeks * light	0.559	3	0.186	0.295	N.S.
NAA * light	148.017	8	18.502	29.278	<.001
weeks * NAA * light	51.941	24	2.164	0.0369	N.S.
Error	136.500	216	0.6319		
Total	3380.247	287			

Table 183.

Mean callus diameter after NAA and dark or light pretreatment and incubation on medium containing 2iP : Rhododendron concinnum.

2iP	Callus diameter (mm)	
mg l^{-1}	dark	light pretreatment
0	0	0
0.1	0	0
0.5	0	0
1.0	0	0
2.5	0	0.25
5.0	2.13	4.13
10.0	2.88	4.75
15.0	3.25	3.88
20.0	2.63	2.63
Mean	1.208b	1.736a

Means followed by different letters are significantly different ($p<.05$)

Table 185.

Analysis of variance for data given in Table 183 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
light	14.222	1	14.222	40.157	<.001
2iP conc	182.299	8	22.787	64.341	<.001
Interaction	22.840	8	2.855	8.061	<.001
Error	19.125	54	0.354		
Total	238.486	71			

Table 184.

Mean callus diameter after 2iP and dark or light pretreatment and incubation on medium containing NAA : Rhododendron concinnum.

NAA	Callus diameter (mm)	
mg l^{-1}	dark	light pretreatment
0	0	0
0.1	0	0
0.5	2.88	7.75
1.0	6.75	11.38
2.5	8.38	13.88
5.0	9.00	9.38
10.0	6.63	0
15.0	0	0
20.0	0	0
Mean	3.736b	4.708a

Means followed by different letters are significantly different ($p<.05$)

Table 186.

Analysis of variance for data given in Table 184 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
light	17.014	1	17.014	13.687	<.001
NAA conc	1352.444	8	169.056	136.000	<.001
Interaction	221.861	8	27.733	22.310	<.001
Error	67.125	54	1.243		
Total	1658.444	71			

Table 187.

Mean callus diameter after dark/light pretreatment followed by light or dark incubation on medium containing 2iP : Rhododendron concinnum.

2iP	Callus diameter (mm)	
mg l^{-1}	dark	light pretreatment
0	0	0
0.1	0	0
0.5	0	0
1.0	0	0
2.5	0	1.63
5.0	3.63	5.50
10.0	3.00	5.75
15.0	1.50	3.38
20.0	1.50	2.63
Mean	1.069b	2.097a

Means followed by different letters are significantly different ($p<.05$)

Table 189.

Analysis of variance for data given in Table 187 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
light	19.014	1	19.014	39.490	<.001
2iP conc	226.000	8	28.250	58.673	<.001
Interaction	17.986	8	2.248	4.669	<.001
Error	26.000	54	0.4815		
Total	289.000	71			

Table 188.

Mean callus diameter after dark/light pretreatment followed by light or dark incubation on medium containing NAA : Rhododendron concinnum.

NAA	Callus diameter (mm)	
mg l^{-1}	dark	light pretreatment
0	0	0
0.1	0	0
0.5	0	0
1.0	1.00	4.25
2.5	4.50	4.88
5.0	1.25	1.25
10.0	0	0
15.0	0	0
20.0	0	0
Mean	0.75b	1.15a

Means followed by different letters are significantly different ($p<.05$)

Table 190.

Analysis of variance for data given in Table 188 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
light	2.920	1	2.920	5.449	<.05
NAA conc	178.236	8	22.280	41.576	<.001
Interaction	18.486	8	2.311	4.312	<.001
Error	28.938	54	0.5359		
Total	228.580	71			

Plate 17.

Callus formation from shoot explant of Chaenomeles japonica - IBA treatment - light incubation.

Plate 18.

Callus formation from shoot explant of Prunus tomentosa - IBA treatment - dark incubation.

5.14 DISCUSSION

All of the species studied required the addition of a growth regulator for callus formation. All species formed callus when auxin (IBA) was supplied. Some species also produced callus in the presence of exogenous cytokinin only. Thus, all species tested can be classified in the first category of Yeoman and Macleod (1977) - 'tissues requiring only an auxin'. 16 species can also be classified in their second category - 'tissues requiring only a cytokinin'.

It is noteworthy that callus formation was promoted in so many species by both auxin and cytokinin. This shows that in these species, dedifferentiation is easily triggered.

Auxins in callus initiation

2,4-D is the most frequently employed auxin for callus initiation from explants of both woody and herbaceous species and is usually active in the concentration range 10^{-5} to 10^{-7} M (Gamborg et al., 1976; Yeoman and Macleod, 1977). Steward and Caplin (1962) found the optimum concentration for carrot explants to be 2.7×10^{-6} M 2,4-D and Murashige and Skoog (1962) used 10^{-6} M for tobacco pith explants. In

woody species, callus formation occurred in the presence of 1.0 ppm (4.5×10^{-6} M) 2,4-D in Populus (Riou et al., 1975), 10^{-6}M in Choisya (Chenieux et al., 1977), 2ppm (9×10^{-6} M) in Rhododendron (Robenek, 1977) and 0.1 ppm (4.52×10^{-7} M) in Vaccinium (Nickerson and Hall, 1976).

The experiments detailed here show 2,4-D to be active in the range 0.1 to 5.0 ppm for Rhododendron concinnum with maximum callus formation occurring at 0.5 ppm. This is comparable with the examples outlined above.

NAA, NOA (2-naphthyloxyacetic acid), IBA and IAA are commonly used but have less activity in callus initiation than 2,4-D (Murashige, 1974; Nickerson and Hall, 1976). Robenek (1977), however, reported greater stimulation of callus formation by IAA than 2,4-D in Rhododendron 'Scarlet Wonder' and Rhododendron wardii X yakusimanum. IAA was not tested in the current experiments but the order of effectiveness in promotion of callus formation, both in terms of number of cultures in which callus formed and callus diameter, was found to be 1) 2,4-D, 2) NAA, 3) IBA in Rhododendron concinnum.

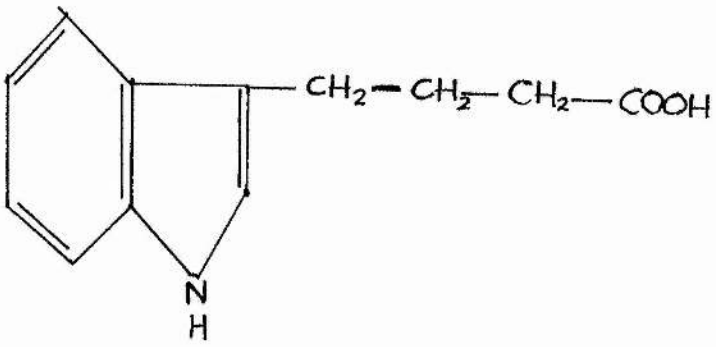
The reasons for this difference in activity could

be related to structure and transport of the different auxins. 2,4-D exhibits only weak polar transport (McCready and Jacobs, 1963), whereas the other auxins used can be transported basipetally more rapidly. However, if absorption occurs principally at the cut basal end of the explant (see Section 5.2), none of the exogenously applied auxins could be transported basipetally. Acropetal transport of auxins in shoots does occur, however (McCready, 1968), but to a much lesser extent. Therefore, little movement of auxin from the site of absorption would take place. Although little information about differences between auxins in acropetal transport rate in shoots has been reported in the literature, it seems unlikely that differences in transport would account for the observed differences between auxins in callus formation.

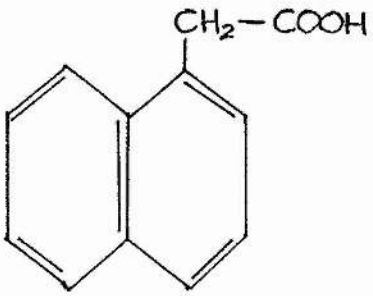
Molecule structure is important in determining to which sites auxin can bind and thus activity of the auxin. Many aspects of auxin structure have previously been studied in relation to physiological effects observed (Schneider and Wightman, 1978). Two aspects are particularly important in considering the different activities of the auxins used in the current experiments. (1) The number of bridging carbon atoms

AUXIN STRUCTURE

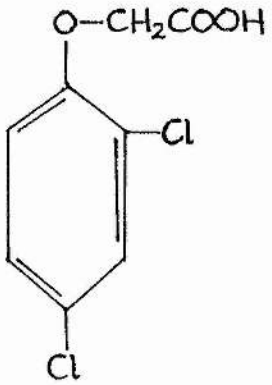
IBA



NAA



2,4-D



between the ring and the carboxyl group determines activity, compounds with one bridging carbon atom showing highest activity (Schneider and Wightman, 1978). IBA has more than one carbon atom and this could account for its lower activity in callus formation than NAA. (2) Nuclear substitution in the para-position of the ring, as in 2,4-D, leads to high activity (Wain and Wightman, 1953).

The duration of exposure to auxin necessary for callus formation was 4 to 7 days when NAA was supplied. An increased exposure time did not affect the number of cultures forming callus. This may show that (1) all of the NAA in the medium is absorbed in 7 days, or (2) that there is a limit to the quantity of NAA which the explant can absorb and this limit is reached by 7 days, or (3) that further uptake of NAA after 7 days does not affect callus formation.

Most visible callus formation occurred between weeks 2 and 4 when IBA and NAA were supplied. Callus formation on medium containing 2,4-D was more rapid (1 to 3 weeks). This may account for the greater quantity of callus formed on medium containing 2,4-D (see above). Callus was initiated more quickly by 2,4-D and therefore had a longer period after initiation in which

to grow.

Nickerson and Hall (1976) reported swelling of the stem explants of Vaccinium when cultured on media containing 2,4-D. They reported that the swelling was due to cell division in the cambial region and enlargement of the cortical cells. This did not occur when alternative auxins were used. Such swelling was also observed in the current experiments and was particularly marked at the cut basal end of the stem explant.

Species differences were observed with respect to callus formation, both in the auxin concentration required for callus initiation and in the range of concentration which promoted callus formation. Other workers have noted that optimal growth regulator concentrations for callus formation vary depending on the species (Gresshof, 1978). In most species, the range of concentration over which callus formation was stimulated was very great. This shows that auxin concentration is unlikely to be a trigger of dedifferentiation.

Appearance of callus varied with the auxin applied. Callus formed on medium containing 2,4-D was flatter than callus on IBA or NAA. The growth

parameter used for recording the results of the experiments was callus diameter. Callus diameter was the parameter used by Nickerson and Hall (1976) in their experiments with Vaccinium callus, but others have used fresh or dry weight. Callus diameter was selected for the current experiments because destruction of the callus was not involved and the callus was available for use in future experiments. However, measurements of diameter take no account of the type of callus produced, i.e. whether the callus was flat or lumpy and so comparisons cannot be made regarding total volume or dry weight of callus formed. However, in spite of the difference in callus form, more callus (by volume) appeared to be formed in 2,4-D treatments than in NAA or IBA treatments.

Others have also noted differences in callus appearance with auxin applied. Robenek (1978) found that IAA induced compact callus growth whereas 2,4-D caused intensive growth with loosely connected cell aggregates in Skimmia, and Mollard et al. (1974) obtained several different tissue types by varying the IAA concentration. 2,4-D promotes cell division in addition to cell elongation (Witham, 1968), whereas the other auxins tested are only known to promote cell

elongation. This may account for some of the difference in growth habit.

Cytokinin activity in callus formation

Cytokinins are frequently added to media for callus formation in combination with auxin and in many cases enhance callus formation (Gamborg et al., 1976). However, they have rarely been used in the absence of auxin for callus induction although species which produce callus in response to cytokinin alone have been reported (Yeoman and Macleod, 1977). Four cytokinins are commonly used :- BA, kinetin, 2iP and more infrequently, zeatin. Effective concentrations are in the range 10^{-6} to 10^{-7} M. 2iP was shown to be the most active in callus formation by Murashige (1974), while other workers have found zeatin to be most active in tobacco callus formation (Leonard et al., 1978).

In the current experiments, callus did not form in the presence of BA in Rhododendron concinnum, and kinetin was less active than 2iP in callus formation. Possible reasons for differences in activity between BA and 2iP in Ericaceous species were discussed in Chapter 3. Murashige (1977) found that 2iP was more active than kinetin in callus formation and this was also found in the current experiments. This difference

in activity is unlikely to be related to cytokinin oxidase activity (discussed in Chapter 3) as 2iP is a substrate for the enzyme but kinetin is not (Whitty and Hall, 1974). Levels of 2iP available for callus formation would be reduced by the oxidase but levels of kinetin would not be reduced in this way; thus kinetin would be expected to have higher activity than 2iP. The reverse was observed. However, kinetin is degraded by cleavage of the N_6-substituent in some plant tissues (Miernyk and Blaydes, 1977) by an as yet unknown enzyme system (Letham and Palni, 1983). Degradation of kinetin could therefore proceed to a differing extent to 2iP degradation depending on the presence of the two enzyme systems in Rhododendron.

Kinetin is not readily transported from the site of application (Lagerstedt and Langston (1967) unless it enters the transpiration stream and therefore would be present at the callus initiation site (also the absorption area) at a high concentration. It is possible that high concentrations of kinetin are inhibitory to callus formation and therefore movement away from the site of absorption or degradation may reduce the cytokinin level to one appropriate for callus initiation. However, when lower concentrations

of kinetin were applied, less callus initiation occurred, thus refuting this proposal.

There was no difference in time for visible callus formation to occur between 2iP and kinetin. Callus was formed in both cases in the third week of culture. Time of formation does not therefore explain the difference in quantity of callus formed by these two cytokinins.

Callus was formed on medium containing 2iP and on medium containing BA in only seven species and cultivars. Of these, three were Ericaceae and four were Rosaceae. There was very little difference in activity between BA and 2iP in these species. This shows that degradation of 2iP by cytokinin oxidase (as discussed in Chapter 3) does not decrease its activity in callus formation in comparison with BA as was proposed to occur during shoot formation (Chapter 3). However, the remainder of the species forming callus on medium containing cytokinin, responded to 2iP if they belonged to the family Ericaceae and to BA if they were Rosaceae (with the exception of Cotoneaster which only formed callus with 2iP). These two cytokinins, therefore, appear to only act on species with particular genetic characteristics indicating that,

with some exceptions, receptor molecules for these two cytokinins may be different and that these receptors could be species specific.

More callus was formed on medium containing auxin than on medium containing cytokinin. When cytokinin only was supplied, lack of auxin may limit cell enlargement and thus callus growth would be less, as observed, than when auxin was present.

Callus formed on medium containing intermediate levels of cytokinin but no auxin was green in colour and contained chloroplasts. This type of callus resulted only in Rosaceae on medium containing BA. Brown callus (possibly lignified) formed in Rosaceous species at high and low BA concentrations. This shows that the concentration range over which BA can promote chloroplast formation is narrow. Chlorophyll synthesis in callus has been reported to occur in other species when callus growth rate is decreased (Laetsch and Stetler, 1965; Stetler and Laetsch, 1968). The effect of BA on chlorophyll synthesis could therefore be indirect via BA-controlled callus growth rate, but BA has also been shown to have a direct stimulatory effect on chlorophyll formation (Fletcher et al., 1973). BA concentration was much more crucial to chloroplast

formation / degradation than to callus formation. This could indicate that concentration acts as a trigger for chloroplast formation / degradation but does not trigger callus formation.

Brown callus also formed in Ericaceous species at all BA concentrations. This may show that BA molecules can probably act at more than one site in Rosaceae (to promote callus and chloroplast formation) whereas in Ericaceous species, BA molecules only act at one site to promote callus formation. Alternatively, the concentration of BA which can trigger chloroplast formation (possibly intermediate between concentrations given) was not supplied. However, callus was green at all 2iP concentrations supplied in both Ericaceae and Rosaceae. This shows that 2iP and BA act differently in chloroplast formation and 2iP concentration probably does not act as a trigger of chloroplast formation.

In several Ericaceous species, 2iP gave a mixed green and red callus. The colours may represent two distinct cell lines similar to those reported by Alfermann and Reinhard (1971) for carrot cultures - anthocyanin synthesising and non-synthesising cell lines. Winton and Mathes (1973) found that presence of sucrose led to the formation of red callus in aspen

whereas sucrose omission resulted in green callus. A gradient in sucrose concentration within the callus might therefore result in a mixed callus.

Auxin and cytokinin interaction

Callus was formed in all species in response to either a cytokinin or an auxin. Since both cell division and cell enlargement are involved in the formation and growth of callus, both auxin and cytokinin probably must be present for callus to form. As auxin and cytokinin applied individually can cause callus formation, one of two possible things may be happening : 1) the additional cytokinin or auxin necessary is obtained from the stem explant and is already present in sufficient quantities or 2) the additional cytokinin or auxin is synthesised in either the developing callus or the stem explant.

It has been shown that tobacco callus which requires auxin for growth, can grow in the absence of auxin when supplied with kinetin (Syono and Furuya, 1972). This callus synthesised IAA. Other workers have also reported an increase in auxin content following cytokinin application. Jordan and Skoog (1971) showed that more auxin was released into agar

from tips of Avena coleoptiles treated with cytokinin than from untreated tips; Saleh and Hemberg (1980) showed that treatment with kinetin increased the level of extractable IAA in Phaseolus, mostly in the primary leaves where most of the kinetin had been absorbed; and Einset (1977) showed that exogenously applied cytokinin decreased the auxin requirement in tobacco tissue cultures.

This increase in IAA content after cytokinin application has been shown to be the result of a decrease in IAA degradation. Cytokinins can inhibit the conversion of IAA to IAA acetylaspartate (Lau, John and Yang, 1977) - IAA acetylaspartate is a virtually inactive conjugate of IAA and cytokinins have also been shown to decrease IAA oxidase activity. Kinetin has been shown to activate IAA oxidising enzymes in roots of Lens culinaris and thus decrease IAA content (Gaspar and Xaufflaire, 1967); and a similar increase in IAA oxidase activity was recorded in barley seedlings (Gaspar et al., 1969). However, Jain et al. (1969) reported that kinetin decreased IAA oxidase activity in dwarf beans and Lee (1971) found that although cytokinins (kinetin and zeatin were tested) in low concentrations (0.2 μM) increased the activity of

IAA oxidase in tobacco callus cultures, higher concentrations (2 to 5 μM) decreased the activity of the oxidase. The effect of cytokinin on IAA oxidase activity and thus on IAA action may therefore depend on cytokinin concentration. Kinetin has also been shown to direct the transport of auxin in leaves of Nicotiana (Lagerstedt and Langston, 1967).

The above reports show that cytokinin can increase or decrease the activity of auxin. It is therefore possible that in species requiring only a cytokinin for callus formation, IAA content is increased by the exogenous cytokinin, and therefore, cytokinin is enhancing auxin activity rather than acting to promote callus initiation independently. Cytokinin and auxin-induced callus was formed in a similar time period. This suggests that an intermediate process is not occurring. However, the time intervals between which callus formation was recorded were of the order of several days and this is probably not a fine enough time scale to detect such differences.

When auxin and cytokinin were applied together simultaneously, more callus formed than when auxin and cytokinin were applied singly. Cytokinins are very important in callus initiation but auxins are necessary

for growth of callus. This was shown by the experiment in which auxin was given as a pretreatment and cytokinin only was supplied in the medium - little callus growth occurred. On the other hand, cytokinin pretreatment with auxin present in the medium resulted in considerable callus growth. Therefore, the sequence of events is important. This experiment shows that cytokinins are necessary for callus initiation and callus formation can be divided into two processes:- (1) cytokinin-stimulated cell division, followed by (2) growth of callus.

It is difficult to separate the phases of initiation and growth. The presence of a large amount of callus at the end of the culture period may indicate that (1) many stem cells have dedifferentiated or (2) only a few stem cells have given rise to callus but this callus has subsequently grown from other callus cells. Section 5.3 investigates callus growth in the absence of the shoot explant to clarify this distinction.

Environmental factors in callus formation

Temperature

Of the temperatures tested, 25° C was most favourable for callus initiation.

At high temperature (33°C), callus of the type formed on medium containing 2,4-D was formed. 2,4-D at high concentrations inhibits hydrogen ion extrusion and potassium ion uptake and inhibits respiration, resulting in a decrease in cell enlargement (Colombo and Ferrari-Bravo, 1982). This type of callus growth may therefore result principally from cell division rather than cell expansion. High temperature (above 28 C) reduces cytokinin levels (Skene and Kerridge, 1967) and lack of cytokinin may affect callus form but would be expected to decrease cell division. However, cytokinin promotes potassium uptake (see Chapter 6) and the ratio of potassium to magnesium has been shown to change friability of callus (Yoshida and Watanabe, 1971). The fact that high temperature also led to the formation of this type of callus may indicate that a phytotoxic response has been invoked - high temperature (30 C) increases the phytotoxicity of auxin herbicides (Harvey and Muzik, 1973). Ethene synthesis occurred after 2,4-D treatment of tobacco tissue cultures and

declined in rapidly growing cultures but remained high in moderately and severely 2,4-D stressed (>0.5 mg l^{-1})cultures (Garcia and Einset, 1983). This type of callus may therefore result from stress, as it was promoted by both 2,4-D and high temperature.

Light

Light interacts with both auxins and cytokinins but in different ways in the species tested. Light inhibits callus formation in the presence of auxin, but promotes callus formation in the presence of cytokinins. Alternatively, darkness may be inhibiting callus formation in the presence of cytokinins and promoting callus formation in the presence of auxins.

Fraser et al. (1967) and Yeoman and Davidson (1971) reported inhibition of callus induction by light in cultured explants from Jerusalem artichoke tubers, and Nickerson and Hall (1976) showed that light inhibited callus formation in the presence of IAA or NAA or NOA in Vaccinium. Chong and Taper (1974) showed that callus of different cultivars of Malus grew differently in relation to light - in this case, the growth of callus as affected by light was related to differing carbohydrate and sorbitol content of the tissues. Carbohydrate content is unlikely to be

important in the experiments under consideration, however, as carbohydrate was supplied in the nutrient medium. The differences in response to light were observed in the same species and were related to the presence of auxin and cytokinin.

Light can reduce IAA concentration in plant tissue by photo-oxidation and increase the quantity of IAA present as esterified forms (Bandurski et al., 1977), but IBA, NAA and 2,4-D are less destructible Posthumus, 1971). Therefore, if total auxin concentration is important for callus initiation, the reduction in IAA would be compensated for by increased exogenous auxin provided to the explant. However, auxin-stimulated callus initiation was not promoted in light even at high exogenous auxin concentrations. Therefore, IAA inactivation is unlikely to be responsible for the observed difference.

The role of light in differentiation and dedifferentiation is discussed further in Chapter 9.

5.2 THE ROLE OF ENDOGENOUS FACTORS IN CALLUS FORMATION

In the previous section, it was shown that exogenously applied auxins and cytokinins promoted callus formation when applied singly as well as in combination. However, the extent to which endogenous growth regulators and other endogenous factors are involved is unclear. This section, therefore, examines the role of the shoot explant in callus formation.

Experiments were conducted to examine the following variables in relation to exogenous auxin concentration and callus formation :- explant size, polarity of explant, presence of shoot apex, presence of axillary buds, derivation of explant, and seasonal variation.

5.21 THE EFFECT OF THE SHOOT EXPLANT ON CALLUS FORMATION.

Stem explants of Rhododendron concinnum were used for the following experiments. NAA was incorporated into the nutrient medium at 0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0 or 2o mg l^{-1}. Number of cultures in which callus was formed was recorded at the end of a four week incubation period in darkness.

Experiment 1 : Explant size.

Explants of the following lengths were cultured in June or December :- 1, 3, 5, 10, 20, 30, 40 or 50 mm.

Experiment 2 : Polarity of explant.

Stem explants with the apex removed (top 3 mm) were placed on the medium in one of three orientations:- 1) flat on medium, 2) vertically not inverted or 3) vertically but inverted.

Experiment 3 : Presence of shoot apex.

The apex was 1) left intact or 2) excised (top 3 mm removed). Explants were then trimmed to 15 mm lengths.

Experiment 4 : Presence of axillary buds.

Stem explants with buds removed to give the following bud numbers were cultured :- 0, 1, 2, 3, 4, 5 or 6.

Experiment 5 : Derivation of explant.

Explants from the top and base of the parent plant were tested for their potential for callus initiation. Stems (50 mm long with the apical 3 mm removed) were divided into five 10 mm long explants.

Results

Experiment 1 : Explant size

Number of cultures in which callus formed is given in Table 191. A higher exogenous auxin concentration was necessary for callus initiation in short explants than in long explants. In short explants, callus formed in a narrow range of exogenous NAA concentrations, whereas the range of effective concentrations was wider for long explants. No callus was formed when explants were shorter than 5mm.

Experiment 2 : Polarity of explant

Number of cultures in which callus formed and callus diameter are given in Table 192. Fewer cultures formed callus when the explants were orientated vertically, and significantly less callus formed ($p<.05$). Callus formation was significantly more ($p<.05$) at the morphological basal end than at the apical end (Plate 19).

Experiment 3 : Presence of shoot apex

Callus diameter at the basal end of explants with the apex excised or intact is given in Table 193. There was no significant difference in callus diameter between cultures with and without the shoot apex (Table 194). However, some callus was also formed at the apical cut end of explants which had the apex excised. These explants therefore, had greater total callus formation.

Explants with the apex excised required a higher exogenous auxin concentration for callus formation than those with the apex intact.

Experiment 4 : Presence of axillary buds

Number of cultures in which callus formed is given in Table 195. A significant ($p<.05$) increase in number of cultures forming callus was shown with increasing bud number (Table 196). There was also a significant effect due to NAA concentration ($p<.001$).

Experiment 5 : Derivation of explant

Callus diameter is given in Table 197. Significantly more callus was formed in explants from the top of the plant than in basal explants ($p<.05$). There was no difference, however, in callus formation between explants within each 50mm length. Explants

from the base of the plant required a higher NAA concentration for callus formation than explants from the top of the plant.

Table 191. Number of cultures (of 40) in which callus was initiated as a function of explant length : Rhododendron concinnum.

Explant length mm	Callus number
1	0a
3	0a
5	10b
10	12b
20	9b
30	8b
40	11b
50	12b

Means followed by different letters are significantly different ($p<.05$).

Number of cultures (of 40) in which callus was initiated as a function of explant length : Rhododendron concinnum.

NAA mg l^{-1}	Callus number
0	0c
0.1	1bc
0.5	3b
1.0	18a
2.5	24a
5.0	12a
7.5	4b
10.0	0c
15.0	0c
20.0	0c

Means followed by different letters are significantly different ($p<.05$).

Table 192. Mean callus diameter from explants from the morphological apex or base when placed vertically or horizontally in Rhododendron concinnum.

Treatment	Callus diameter mm
Horizontal	0.833a
Vertical	0.250b
Vertical inverted	0.0694

Mean callus diameter from explants from the morphological apex or base when placed vertically or horizontally in Rhododendron concinnum.

Treatment	Callus diameter mm
Apex	14.5b
Base	68.5b

Effect of concentration of NAA mg l^{-1}.

NAA mg l	Callus diameter
0	0c
0.1	0c
0.5	0c
1.0	0c
2.5	0.958b
5.0	1.958a
7.5	0.542b
10.0	0c
15.0	0c
20.0	0c

Means followed by different letters are significantly different ($p<.05$).

Table 193.

Mean callus diameter after 4 week incubation period on auxin containing media : apex excised or intact : Rhododendron concinnum.

NAA	Callus diameter (mm)	
mg l^{-1}	+ apex	- apex
0	0	0
0.1	0	0
0.5	0	0
1.0	4.75	0
2.5	6.25	1.63
5.0	1.63	8.00
10.0	0	3.75
15.0	0	0
20.0	0	0
Mean	2.56	3.09

Table 194.

Analysis of variance for data given in Table 193 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
apex	2.258	1	2.258	0.2040	N.S.
Error	332.047	30	11.068		
Total	334.305	31			

Table 195.

Number of cultures (of 36) in which callus formed with varying bud number on explant in Rhododendron concinnum.

Bud number	Number with callus
0	1
1	1
2	4
3	10
4	7
5	9
6	8

Number of cultures (of 28) in which callus formed with varying NAA concentration in Rhododendron concinnum.

NAA mg l^{-1}	Callus number
0	0
0.1	0
0.5	0
1.0	6
2.5	19
5.0	12
10.0	3
15.0	0
20.0	0

Table 196.

Analysis of data in Table 195.

χ^2 number of cultures with callus and bud number = 14.61 ($p<.05$).

χ^2 number of cultures with callus and NAA concentration = 63.35 ($p<.001$).

Analysis of variance for data given in Table 195 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
bud number	3.9185	6	0.6531	2.7865	<.05
Error	4.9218	21	0.2344		
Total	8.8404	27			

A loge (y+1) transformation was used

Table 197.

Mean diameter of callus formed on explants taken from the top or base of the parent plant : Rhododendron concinnum.

Explant position	Diameter of callus mm	
(see text)	TOP	BASE
1	1.68	1.42
2	1.65	1.24
3	1.42	1.18
4	1.60	1.61
5	1.61	1.11

Mean callus diameter (mm) for explants derived from top or base of parent plant of Rhododendron concinnum.

ORIGIN	Callus diameter mm
TOP	1.592a
BASE	1.311b

Means followed by different letters are significantly different ($p<.05$).

Plate 19.

Callus formation from shoot explant of Rhododendron concinnum - more callus formed at the morphological base.

5.22 SEASONAL VARIATION

Method

Experiment 1. Stem explants of Rhododendron concinnum were cultured in April, June, August, October, December and February on nutrient medium containing NAA at 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}. Number of cultures in which callus formation occurred was recorded at the end of a four week incubation period in darkness.

Experiment 2. The length of time for callus to be initiated was studied in explants cultured in June and December. Number of cultures in which callus was formed was recorded after 2, 4 and 8 weeks of incubation.

Experiment 3. Explants were given a cytokinin quick dip (0.5% 2iP) prior to incubation in June and December on nutrient medium supplemented with NAA at 0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mg l^{-1}. Number of cultures in which callus was formed was recorded at the end of a four week incubation period in light (16 hour photoperiod) or darkness.

Results

Experiment 1.

Number of cultures in which callus formed was plotted against NAA concentration (Figure 414). Callus formation peaked in June and August and was least in winter (Table 198). NAA concentration required for callus formation was less in summer than in winter (Figure 414).

Experiment 2.

Table 199 gives number of cultures in which callus formed in June and December after 2, 4 and 8 weeks of incubation. Callus formed significantly ($p<.05$) more rapidly (within two weeks of explantation) in June-cultured explants than in December-cultured explants (5 to 8 weeks).

Experiment 3.

When a 2iP quick dip was given prior to culture on medium containing NAA, significantly more ($p<.05$) explants formed callus in both June and December than in non-pretreated explants (Table 200). The range of effective NAA concentrations was also greater in

cytokinin-pretreated explants.

Table 198.

Number of cultures (of 4) in which callus formed when explanted in April, June, August, October, December or February : Rhododendron concinnum.

	Number of cultures (of 4) with callus						
NAA mg l^{-1}	Month						
	April	June	Aug	Oct	Dec	Feb	Total
0	0	0	0	0	0	0	0
0.1	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0
1.0	0	2	1	0	0	0	3
2.5	1	4	4	2	0	0	11
5.0	3	2	3	2	0	0	10
10.0	2	0	0	2	3	4	11
15.0	0	0	0	0	0	0	0
20.0	0	0	0	0	0	0	0
Total	6	8	8	6	3	4	35

Figure 414.

Percentage of cultures in which callus formed in four week incubation periods : Rhododendron concinnum : explants cultured in different months.

R^2 = .7120

r = -.8438 ($p<.05$)

S.E. = 15.309

Fig 4.4 Rhododendron concinnum.

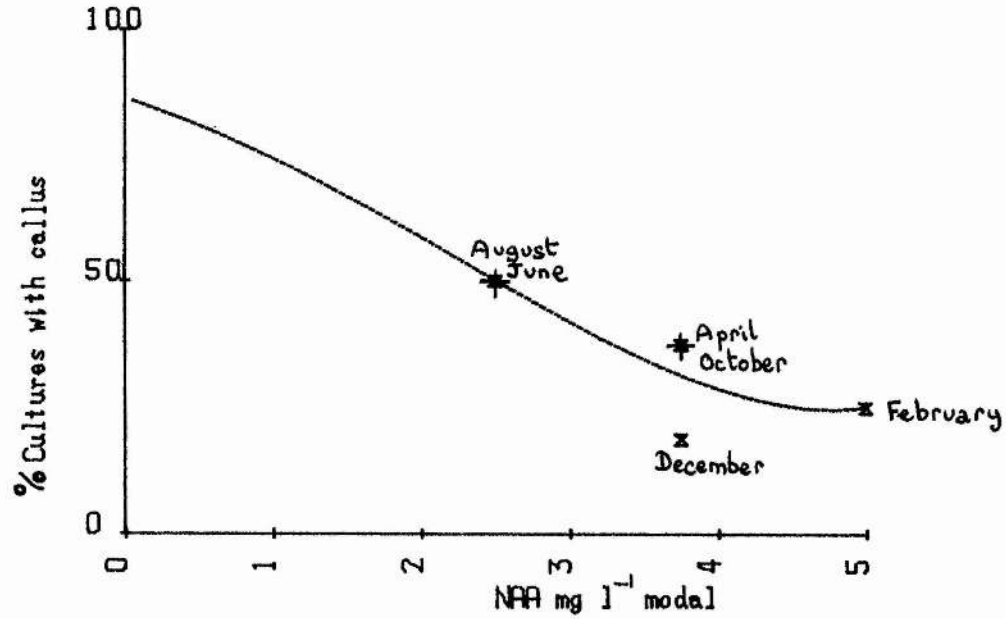


Table 199.

Number of cultures (of 4) in which callus formed after 2, 4 and 8 weeks cultured in June and December : Rhododendron concinnum.

NAA mg l^{-1}	Number of cultures (of 4) with callus						
	JUNE			DECEMBER			
	WEEKS						
	2	4	8	2	4	8	Total
0	0	0	0	0	0	0	0
0.1	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0
1.0	2	2	2	0	0	0	6
2.5	4	4	4	0	0	1	13
5.0	2	2	2	0	0	1	7
10.0	0	0	0	0	3	4	7
15.0	0	0	0	0	0	0	0
20.0	0	0	0	0	0	0	0
Total	8	8	8	0	3	6	33

Table 200.

Number of cultures (of 4) in which callus formed after incubation on medium containing NAA with cytokinin pretreatment in light or dark : Rhododendron concinnum.

	Number of cultures (of 4) with callus				
NAA mg l^{-1}	JUNE		DECEMBER		
	light	dark	light	dark	Total
0	0	0	0	0	0
0.1	0	0	0	0	0
0.5	4	4	0	0	8
1.0	4	4	0	2	10
2.5	4	4	3	4	15
5.0	4	4	4	4	16
10.0	0	4	2	3	9
15.0	0	0	0	0	0
20.0	0	0	0	0	0
Total	16	20	9	13	58

5.23 DISCUSSION

Explant size

Longer explants required less exogenous auxin for callus initiation than short explants. This demonstrates that lack of auxin may be limiting callus initiation in small explants. Therefore, endogenous auxin content contributes significantly towards initiation of callus.

Polarity of explant

More callus was formed at the morphologically basal end of the explant. This has been shown previously (Gautheret, 1959; Nickerson and Hall, 1976) and could be related to polar transport of auxin. Therefore, endogenous auxin may be important in determining the suitability of sites for callus initiation even when exogenous auxin is present.

Less callus formed when explants were oriented vertically in the medium. This may show that auxin and other factors in the medium are not absorbed to such a great extent - less of the explant is in contact with the medium than in horizontally placed explants. However, Heller (1965) showed that stem segments show

less absorption when laid horizontally on agar (22.7% after 25 days) than in the vertical position (71%). Alternatively, auxin absorbed at the base (normally transported polarly) is not transported to the top of the explant leading to non-formation of callus at the top. Callus may not form at the base of the explant because of lack of oxygen where the explant is embedded in the medium.

Presence of shoot apex

Explants with the apex excised required a higher concentration of exogenous auxin for callus initiation to occur. This shows that the auxin of the apical region may be important in callus formation. However, there was no difference in the quantity of callus formed between explants with and without the apex. This shows that the auxin of the apex is likely to be the only apex factor important in callus initiation.

The difference between auxin concentration required for callus initiation in explants with and without the apex may, therefore, be equivalent to the quantity of free auxin present in the apex. This would be approximately 2.5 mg l^{-1} NAA. NAA is not an endogenous auxin. The IAA concentration which would

produce the same effect on callus formation would probably be greater than this. (NAA at 0.5 mg l^{-1} is equivalent to 10 mg l^{-1} IAA in xylogenesis (Roberts, personal communication).)

Presence of axillary buds

Presence of axillary buds promoted callus formation. These buds contain high concentrations of auxin which, if released, may promote callus formation. By removing the buds, a source of auxin is also being removed. However, The same concentration of NAA promoted callus formation regardless of the number of buds present. Therefore, the auxin content of the buds is unlikely to be important in controlling callus formation. Some other bud factor is therefore affecting callus formation. This factor could be sucrose (see Chapters 4 and 6). The effect of buds on differentiation is discussed in Chapter 4. This discussion is also relevant to these results.

Derivation of explant

Callus was initiated more readily from explants from the top of the plant than from explants from the base of the plant. A higher NAA concentration was

required for basal explants than for explants from the top of the plant. These results may reflect a difference in auxin concentration from top to bottom of the plant - see discussion Chapter 4.

Seasonal variation

Callus formed more readily in summer than in spring or winter. A higher auxin concentration enhanced callus formation in winter. This shows that either endogenous auxin content varies with season (less being present in winter) or other factors in the shoot explant vary with season and these factors determine response to auxin. Seasonal variation in endogenous growth regulator content is discussed in Chapter 4.

Callus formation occurred more rapidly in summer than in winter. The fact that callus formed, shows that requirements in terms of growth regulators and nutrients have been met. The difference in rate may therefore reflect a difference in energy supply for dedifferentiation. Sucrose was supplied in the medium and therefore, is unlikely to limit rate of callus formation in winter but not in summer. The difference may lie in the rate at which the sucrose is

metabolized, and the rate at which other reactions involved in callus formation take place. Incubation conditions were similar at both times of year and therefore, rates may be limited by internal factors of the explants rather than, for example, temperature of incubation. Alternatively, callus formation in winter may involve more intermediate processes than in summer.

Cytokinin given as a pretreatment before incubation on medium containing NAA, promoted callus formation in both June and December suggesting that endogenous cytokinin may be limiting for callus formation at both times of year. However, cytokinin pretreatment did not change the fact that explants cultured in December produced less callus than those cultured in June. However, only one 2iP concentration was applied. As endogenous cytokinin concentration varies with season (see Chapter 4), different exogenous cytokinin concentrations may be required at different times of year to produce the same degree of callus formation.

5.3 CALLUS GROWTH

In Section 5.1 it was found that it was difficult to distinguish the factors responsible for callus initiation from those responsible for subsequent growth of the callus. This section investigates callus growth in the absence of the shoot explant to determine the growth regulator requirements for callus growth. By comparing results from this section with results from Section 5.1, it should be possible to distinguish between factors responsible for growth and those necessary for dedifferentiation.

Callus growth has received considerable attention in a few species. Tobacco and soybean callus are used extensively in cytokinin bioassays (Linsmaier and Skoog, 1965; Miller, 1963). In their classic paper, Skoog and Miller (1957) reported the effects of different concentrations and combinations of exogenous auxin and cytokinin on tobacco callus growth and morphogenesis. However, other workers (Street, 1977; Hussey, 1978; Steward and Krikorian, 1979) have shown that the Skoog and Miller example is an oversimplification of the case and that other species respond differently.

Auxin activity in callus growth was therefore investigated in Rhododendron concinnum, a representative species of the Ericaceae. In section 5.1, it was shown that different callus types were produced as a result of different growth regulator treatments. This section studies these different callus types to determine whether the types remain distinct when cultured a) in the absence of the shoot explant, b) under different growth regulator conditions and c) on solid or liquid media, i.e. whether the conditions of callus initiation determine subsequent growth of undifferentiated tissue.

5.31 GROWTH AFTER EXCISION FROM SHOOT EXPLANT

Method

Experiment 1.

To determine whether callus retains its character in the absence of the shoot explant, callus (3mm diameter) of Rhododendron concinnum was grown on an identical medium to that on which it was initially formed. Three types of callus were selected:- 1) lumpy friable callus, 2) glistening white flat callus and 3) green callus (see Section 5.1 for details of initiation

of these callus types). Callus diameter and appearance were recorded at the end of a four week incubation period. Light treatment was the same as that given for callus initiation.

Experiment 2.

To determine whether callus retains its character when cultured on different media, callus (3mm diameter) of the three types specified in Experiment 1 above of Rhododendron concinnum was cultured on nutrient medium supplemented with NAA at 0, 1.0, 2.5, 5.0 or 10.0 mg l^{-1}. Cultures were incubated in either light (16 hour photoperiod) or darkness for four weeks. Callus diameter and appearance were recorded at the end of this period.

Results

Experiment 1.

There was a significant difference in callus growth between the three types of callus ($p<.001$) (Table 201). Callus of Type (2) grew most rapidly (flat glistening callus) and Type (3) (green callus) grew most slowly. Growth of all three callus types was more rapid in the absence of the shoot explant. At the

end of the four week culture period, callus appearance was similar to that at the beginning of the experiment (Table 207). However, the callus was slightly flatter in all cases.

Experiment 2.

Tables 202 and 203 give mean callus diameter for the three callus types after culture on medium containing NAA in light and in dark. An analysis of variance (Table 204) showed a significant effect ($p<.001$) due to callus type and a significant effect due to NAA concentration ($p<.001$). There was a significant interaction ($p<.001$) between these. However, there was no significant difference between callus growth in light and in darkness.

Growth was more rapid in the absence of the shoot explant. Callus type changed towards Type (1) in all cases. However, the difference in growth rate between the different types of callus shows that there was a carryover effect from the previous treatment. 2,4-D had the most pronounced carryover effect. The optimal concentration for callus growth was lower than when the shoot explant was present.

Table 201

Callus growth and callus type : Rhododendron concinnum.

Callus type	Treatment	Type at end	Diameter inc mm
I Nodular	NAA 2.5 mg	as beginning	7.25b
	1 dark		
II Flat	2,4-D 1.0mg	as beginning	12.50a
glistening	1 dark		
III Green	2iP 10.0 mg	some parts	6.63b
	1 light	not green	

Means followed by different letters are significantlt different ($p<.05$).

Analysis of variance for data given in Table 195 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
Callus typ	83.292	2	41.646	22.802	<.001
Error	16.438	9	1.826		
Total	99.729	11			

Table 202

Callus growth increase in 4 weeks and callus type in Rhododendron concinnum in light (16 hour day).

NAA mg l^{-1}	Callus type		
	I	II	III
0	1.38	2.38	0.5
1.0	6.38	8.25	1.63
2.5	2.13	4.13	1.75
5.0	1.88	1.88	1.00
10.0	2.00	2.38	0.88
L.S.D.	1.68		

Table 203.

Callus growth increase in 4 weeks and callus type in Rhododendron concinnum in darkness.

NAA mg l^{-1}	Callus type		
	I	II	III
0	1.00	2.13	0.25
1.0	3.63	6.63	1.75
2.5	2.88	6.25	2.25
5.0	2.00	4.75	1.13
10.0	1.13	3.13	0.38
L.S.D.	1.68		

Table 204.

Analysis of variance for data in Figures 202 and 203 : Rhododendron concinnum.

SOURCE	S.S.	D.F.	M.S.	F	p
Callus type	185.954	2	92.977	68.801	<.001
NAA conc.	186.325	4	46.581	34.469	<.001
light	0.0750	1	0.0750	0.0555	N.S.
Callus * NAA	47.213	8	5.902	4.367	<.001
Callus * light	9.837	2	4.919	3.640	<.05
NAA * light	26.842	4	6.710	4.966	0.01
Callus * NAA * light	14.621	8	1.827	0.9099	N.S.
Error	121.625	90	1.351		
Total	592.492	119			

5.32 DISCUSSION

Growth of callus was more rapid in the absence of the shoot explant. This may reflect acclimatization of the callus to the culture conditions as discussed in Chapter 3 or alternatively, factors present in the shoot explant may be inhibitory to callus growth.

The growth regulator concentrations which promoted callus growth to the greatest extent in these experiments were lower than those which promoted callus initiation in Section 5.1. Also, callus growth was promoted by a wider range of auxin concentrations than callus initiation. This may indicate that dedifferentiation is controlled by growth regulator concentration whereas callus growth is not.

Callus type was the same after subculture as at the beginning of the experiment when the growth regulator supplied was the same, thus indicating that the shoot explant did not affect callus type.

However, when callus was cultured on medium containing a different growth regulator formulation from the initiation formulation, the callus type changed. Therefore, callus type is determined by the current growth regulator content of the medium. There

was, however, some carryover effect from the previous treatment. This carryover effect was confined to growth rate of the callus rather than callus type.

No difference was shown between callus growth in light and callus growth in dark incubation. Light / dark is therefore less important for callus growth than for callus initiation.

VI. NUTRIENT MEDIA FACTORS IN THE CONTROL OF MORPHOGENESIS

6.1 SUCROSE CONCENTRATION

The presence of an exogenous supply of carbohydrate has been shown to be essential for sustained growth of callus cultures (Street, 1973), and for xylogenesis in cultured tissues (Fosket and Roberts, 1964).

In addition, sucrose has been shown to display some hormone-like effects on growth, for example, treatment with sucrose shortened the lag phase of xylem differentiation (Cawthon, 1972) and stimulated differentiation in callus (Jeffs and Northcote, 1967). It is hypothesised that sucrose interacts with growth regulators in differentiation.

An experiment was therefore conducted to determine (1) whether sucrose is essential for shoot, root and callus formation, and (2) whether sucrose concentration affects morphogenesis.

Method

Shoot explants of the following species were used in the experiment:- *Spiraea* 'Froebelii' and *Arctostaphylos uva-ursi*.

Nutrient medium was prepared as previously described (Section 2.21) but with the following sucrose concentrations :- 0, 10, 20, 30, 40, 50, 60 g l^{-1}. BA, 2iP and IBA were incorporated in the medium at the concentrations which promoted maximal shoot, root or callus development.

Shoot and root number, callus formation and explant growth were recorded at the end of a four week incubation period.

Results

No shoots, roots or callus formed in the absence of sucrose.

Shoot formation

Shoot number is given in Figures415 to 418. An analysis of variance (Table 205) showed a significant effect ($p<.001$) on shoot formation due to sucrose concentration, a significant difference between BA and 2iP ($p<.001$) and a significant difference ($p<.001$) between species. A significant interaction was also demonstrated between sucrose concentration and cytokinin ($p<.01$), and between sucrose concentration and species ($p<.001$). In both species, shoot number

was greatest at a sucrose concentration of 30 g l^{-1}. At high sucrose concentrations (50 and 60 g l^{-1}, leaves had some red pigmentation.

Root formation

Root number was greatest in both species at 30 g l^{-1} (Table 206). No roots formed at sucrose concentrations greater than 40 g l^{-1}. An analysis of variance (Table 207) showed a significant effect on root formation due to sucrose concentration ($p<.001$), to species ($p<.001$) and a significant interaction ($p<.001$) between sucrose concentration and species.

Callus formation

Callus formation occurred in both species at 20, 30 and 40 g l^{-1} sucrose but occurred in fewer cultures at the other concentrations tested (Figures 419 and 420).

Explant growth

Explant growth is given in Tables 208 and 209. In Arctostaphylos, explant growth was increased by sucrose concentrations of 20 to 40 g l^{-1} or more, and in Spiraea explant growth was enhanced by 20 g l^{-1} sucrose.

Figures 415 to 418.

Shoot number at the end of a four week incubation period on medium containing BA or 2iP and varying sucrose concentration.

Figures 419 and 420.

Number of cultures in which callus formed on medium containing varying concentrations of sucrose (recorded after four weeks incubation).

Fig 415. Spiraea 'Froebelii BA.

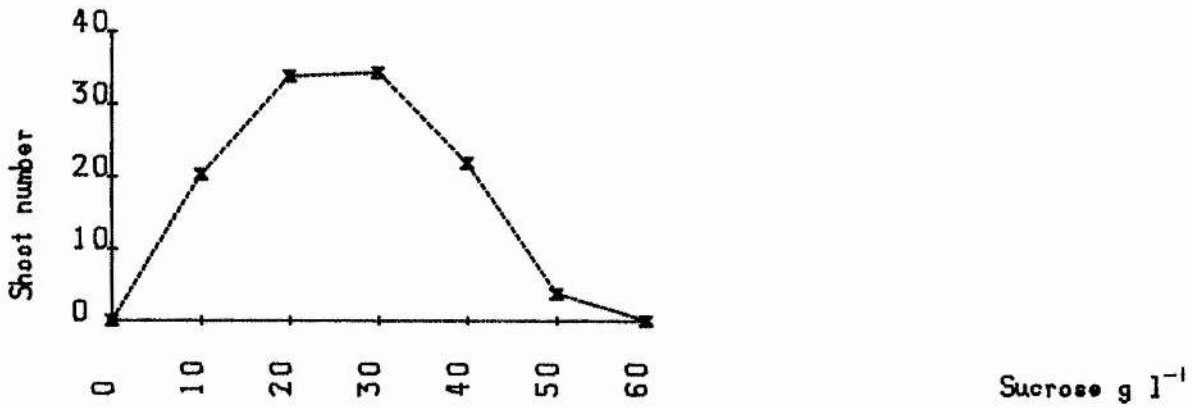


Fig 416. Spiraea 'Froebelii' 21P.

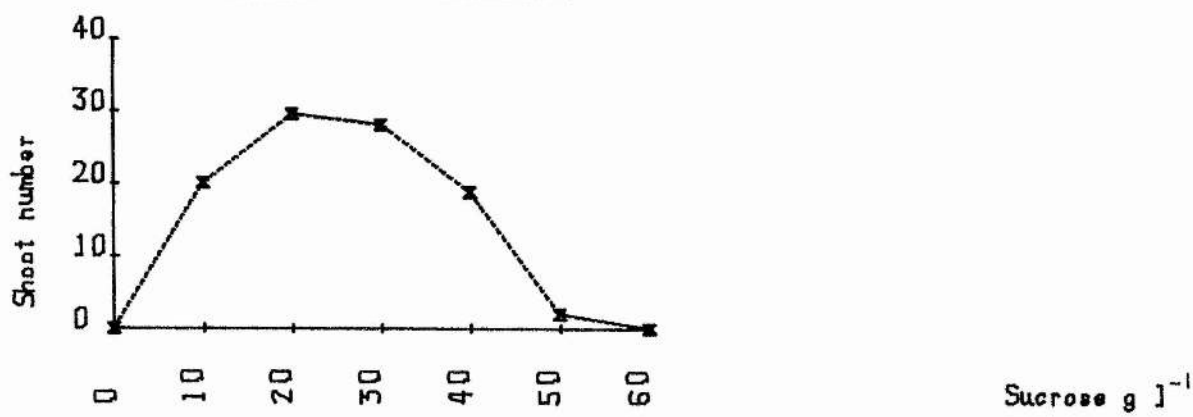


Fig 417 Arctostaphylos uva-ursi BA.

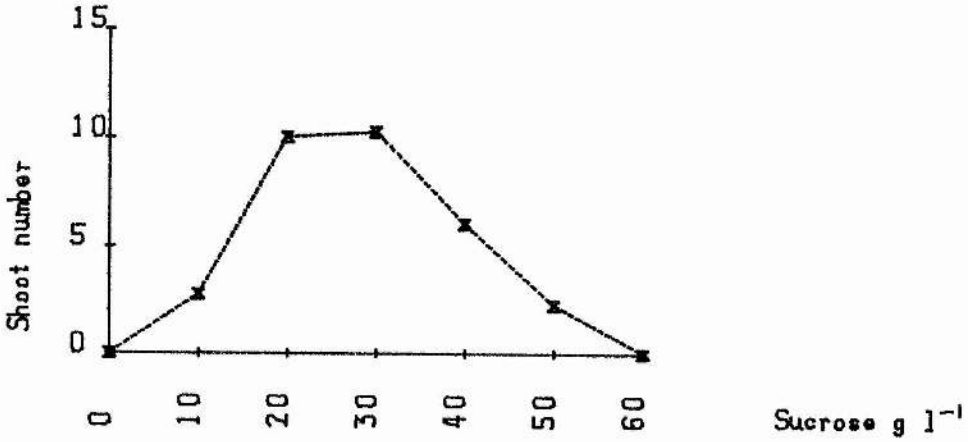


Fig 418 Arctostaphylos uva-ursi 2iP.

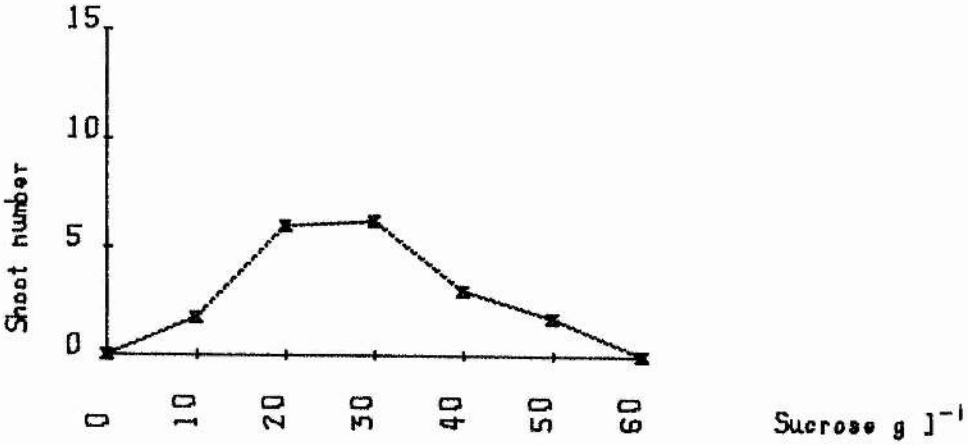


Fig 419. Spiraea 'Froebelli'.

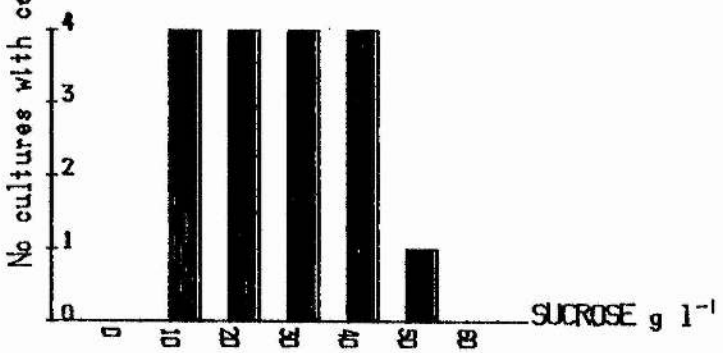


Fig 420. Arctostaphylos.

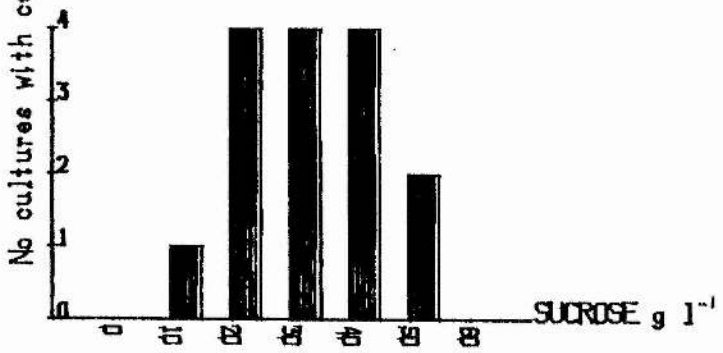


Table 205.

Analysis of variance for data in Figures 415 to 418

SOURCE	S.S.	D.F.	M.S.	F	p
Sucrose conc	7223.714	6	1203.952	219.852	<.001
Growth regulator	112.000	1	112.000	20.452	<.001
Species	3749.143	1	3749.143	684.626	<.001
Sucrose * growth regulator	103.750	6	17.292	3.158	<.01
Sucrose * species	2849.857	6	474.976	86.735	<.001
Growth regulator * species	1.286	1	1.286	0.2348	N.S.
Sucrose * growth regulator * species	5.964	6	0.994	86.735	<.001
Error	460.000	84	5.476		
Total	14505.714	111			

Table 206.

Root number at the end of 4 weeks incubation on medium with varying sucrose concentration.

	Mean number of roots							
Species	Sucrose g l^{-1}							
	0	10	20	30	40	50	60	Mean
Spiraea	0	3.5	105	16.0	13.5	0	0	6.21a
Arctostaphylos	0	2.75	8.75	10.0	7.5	0	0	4.14b
Mean	0d	3.13c	9.63b	13.0a	10.5b	0d	0d	5.18

Means followed by different letters within a column or row are significantly different ($p<.05$).

Table 207.

Analysis of variance of data given in Table 206.

SOURCE	S.S.	D.F.	M.S.	F	p
Sucrose conc	1551.464	6	258.577	78.984	<.001
Species	60.071	1	60.071	18.349	<.001
Interaction	91.179	6	15.196	4.642	<.01
Error	137.500	42	3.274		
Total	1840.214	55			

Table 208.

Explant growth (mm) of Arctostaphylos uva-ursi after culture on media containing growth regulators and varying concentrations of sucrose.

Explant growth range.

Growth regulator	Sucrose g l^{-1}						
	0	10	20	30	40	50	60
BA	1-5	1-5	1-5	1-5	6-10	6-10	6-10
2iP	6-10	6-10	11-15	11-15	11-15	11-15	11-15
IBA	6-10	6-10	6-10	11-15	11-15	11-15	

Table 209.

Explant growth (mm) of Spiraea 'Froebelii' after culture on media containing growth regulators and varying concentrations of sucrose.

Explant growth range.

Growth regulator	Sucrose g l^{-1}						
	0	10	20	30	40	50	60
BA	1-5	1-5	6-10	6-10	6-10	6-10	6-10
2iP	6-10	6-10	6-10	6-10	6-10	6-10	6-10
IBA	6-10	6-10	11-15	11-15	11-15	11-15	11-15

6.2 MINERAL CONTENT

The nutrient media used for the experiments described in this thesis have a high concentration of mineral salts. However, Anderson (1975) reported that shoot explants of Rhododendron did not survive on Murashige and Skoog (1962) medium and noted that KNO concentration appeared to be crucial in determining survival. Other workers have shown that high salt concentrations can be inhibitory to rhizogenesis (Lovell et al., 1971; Khalighy, 1976).

Certain mineral ions are thought to mediate growth regulator responses, for example, K^+, Ca^{2+} (Penny and Penny, 1978). It is hypothesised that mineral availability may play a major role in regulating differentiation.

An experiment was therefore conducted to determine the effect of mineral salt content of the medium on shoot and root differentiation and on callus formation.

Method

Shoot explants of Spiraea 'Froebelii' and Arctostaphylos uva-ursi were used in this experiment.

Nutrient medium was prepared as previously described (Section 2.21) but with one of the inorganic salts omitted (16 treatments). Additional treatment omitted all salts containing potassium. BA, 2iP and IBA were incorporated in the medium at the concentrations which promoted maximal shoot, root or callus development.

Shoot and root number and callus formation were recorded at the end of a four week incubation period.

Results

Shoot formation

Shoot number is given in Table 210. An analysis of variance (Table 211) showed a significant effect on shoot formation due to mineral salt excluded ($p<.001$) and to species ($p<.001$) but no difference between BA and 2iP treatments. In both species, shoot formation was significantly decreased when potassium was omitted

from the medium completely or partially. Omission of other salts did not change shoot number to such a great extent (Table 212), but in some treatments, shoots appeared chlorotic.

Root formation

Root number was increased by the reduction of potassium in the form of KNO_3 but decreased when potassium was totally excluded from the medium (Table 2213. Elimination of magnesium also increased root number. An analysis of variance (Table 214) showed a significant effect ($p<.001$) due to mineral salt excluded and to species, and a significant interaction between these ($p<.001$).

Callus formation

Reduction of potassium in the medium considerably decreased the number of cultures in which callus formed (Figures 421 and 422). Reduction of other inorganic salts did not change the number of cultures in which callus was produced.

Table 211.

Analysis of variance for data given in Table 210.

SOURCE	S.S.	D.F.	M.S.	F	p
Salt	5546.235	16	346.640	42.808	<.001
Growth regulator	25.941	1	25.941	3.204	N.S.
Species	20755.059	1	20755.005	92563.749	<.001
Salt * growth regulator	1473.809	16	92.113	11.378	<.001
Salt * species	3626.191	16	226.637	27.995	<.001
Growth regulator * species	171.529	1	171.529	21.188	<.001
Salt * growth regulator * species	615.971	16	38.498	27.995	<.001
Error	1651.500	204	8.096		
Total	33866.235	271			

Table 210

Mean shoot number after 4 weeks incubation on medium with salts excluded.

Salt excluded	Mean shoot no.			
	Spiraea		Arctostaphylos	
	BA	2iP	BA	2iP
Complete	34.25	28.0	10.25	6.25
NH_4NO_3	30.75	26.0	10.0	6.25
KNO_3	5.75	22.5	4.5	4.75
$CaCl_2$	31.0	26.0	10.75	6.0
$MgSO_4$	24.25	28.75	9.75	7.0
KH_2PO_4	24.0	29.75	6.25	6.0
NaH_2PO_4			9.5	6.0
Na_2EDTA	26.5	28.25	11.5	8.75
$FeSO_4$	32.5	28.5	10.75	6.0
H_3BO_4	30.0	24.25	9.25	6.75
$MnSO_4$	30.25	28.0	10.25	8.0
$ZnSO_4$	33.75	28.5	8.0	7.25
KI	17.5	25.5	4.0	6.75
Na_2MoO_4	31.5	26.75	11.75	6.75
$CuSO_4$	29.29	30.25	10.25	6.5
$CoCl_2$	32.75	27.5	9.25	7.0
$KNO_3+KH_2PO_4+KI$	4.75	24.25	2.75	4.75

Table 212

Mean shoot number after 4 weeks incubation on medium with salts excluded (results combined for species).

Salt excluded	Mean shoot no.	
complete	19.69	a
$CuSO_4$	19.63	a
$FeSO_4$	19.63	a
$ZnSO_4$	19.38	a b
Na_2MoO_4	19.19	a b
$CoCl_2$	19.13	a b
$MnSO_4$	19.13	a b
Na_2EDTA	18.75	a b
$CaCl_2$	18.44	a b c
NH_4NO_3	18.25	a b c
H_3BO_3	18.19	a b c
$MgSO_4$	17.44	a b c
KH_2PO_4	16.50	a b c
KI	13.44	d
KNO_3	9.38	e
KNO_3 $+KH_2PO_4$ $+KI$	9.13	e
NaH_2PO_4	3.81	

Table 213.

Root formation on medium containing IBA when medium constituents omitted.

Salt excluded	Mean root no	
	Spiraea	Arctostaphylos
Complete	18.0d	10.0b
NH_4NO_3	21.0c	12.25a
KNO_3	30.75a	15.0a
$CaCl_2$	17.75d	9.75b
$MgSO_4$	21.25cd	10.25a
KH_2PO_4	23.5c	12.0a
NaH_2PO_4		11.25a
Na_2 EDTA	18.25d	10.5a
$FeSO_4$	20.5c	9.0b
H_3BO_3	18.0d	9.75b
$MnSO_4$	24.5bc	15.25a
$ZnSO_4$	21.25cd	10.75a
KI	29.0ab	12.75a
Na_2MoO_4	18.75d	9.75b
$CuSO_4$	17.0d	7.75b
$CoCl_2$	20.0c	13.25a
$KNO_3 + KH_2PO_4 + KI$	9.0e	5.5c
Mean	19.32a	10.87b

Means within a column or row followed by different letters are significantly different ($p<.05$).

Table 214.

Analysis of variance of data given in Table 213.

SOURCE	S.S.	D.F.	M.S.	F	p
Salt	2353.882	16	147.118	11.762	<.001
Species	2431.066	1	2431.066	194.371	<.001
Interaction	1123.059	16	70.191	5.612	<.001
Error	1275.750	102	12.507		
Total	7183.757	135			

Figures 421 and 422.

Number of cultures in which callus formed in a four week incubation period on media lacking mineral salts.

Fig 424. Callus formation on different media: Spiraea.

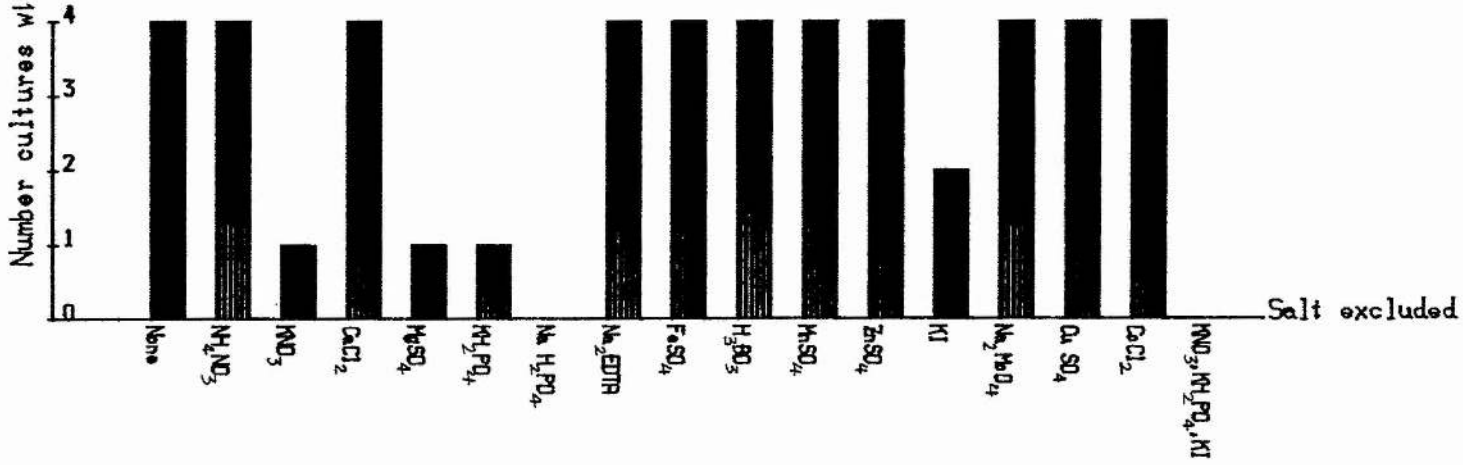
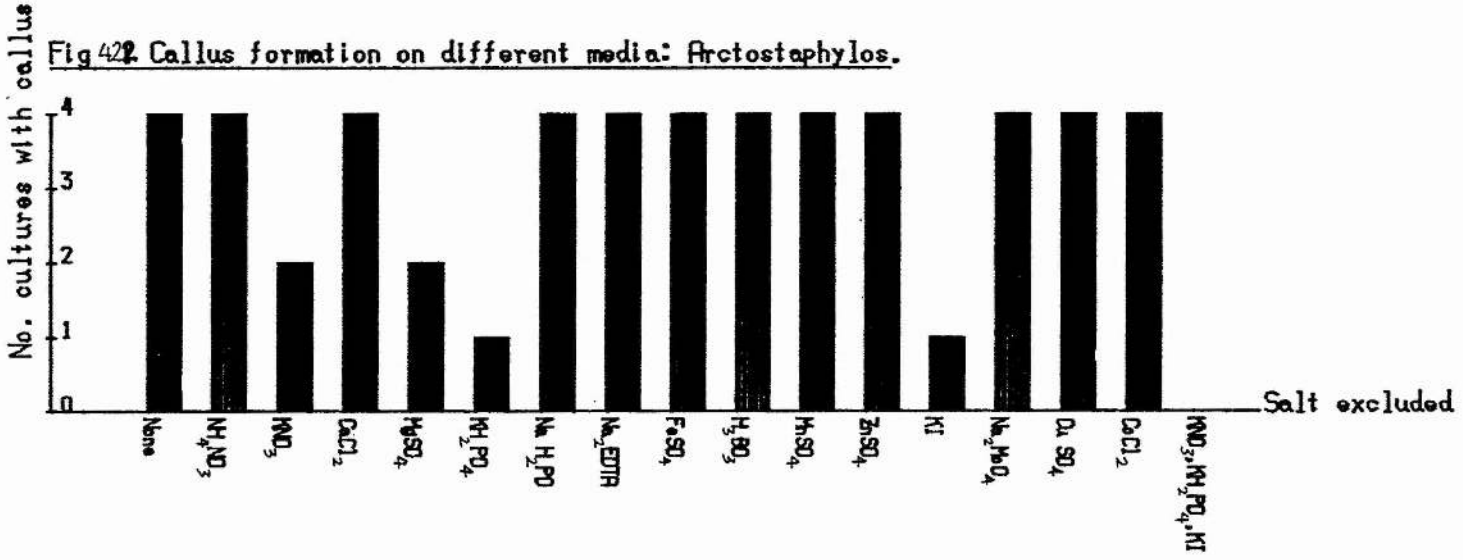


Fig 42. Callus formation on different media: Arctostaphylos.



6.3 HYDROGEN ION CONCENTRATION

While an acid medium has been shown to promote root formation (Hartmann and Kester, 1975), a more alkaline medium favours shoot growth. Hydrogen ion concentration may therefore important in determining direction of differentiation and may mediate growth regulator effects (Penny and Penny, 1978).

In vitro culture of plant organs and tissues is usually conducted at a low medium pH and in the experiments described in this thesis, the pH of the medium was initially set to 4.8 for Ericaceous species and 5.8 for Rosaceous species. These hydrogen ion concentrations would therefore be expected to promote root initiation but not shoot growth.

An experiment was therefore conducted to determine whether a change in pH affects the growth regulator requirements for differentiation.

Method

Shoot explants of Crataegus brachyacantha and Rhododendron 'P J M Victor' were used for this experiment. BA, 2iP or IBA was added to the medium as

follows:-

BA 0, 0.5, 1.0 or 2.5 mg l^{-1} for Crataegus

2iP 0, 10, 15 or 20 mg l^{-1} for Rhododendron.

IBA was added at 0, 1.0, 2.5, 5.0 or 10.0 mg l^{-1}.

The pH of the medium was adjusted with 1N HCl or 1N NaOH to 3, 4, 5, 6, 7, 8, 9 or 10. Agar concentration was increased at pH 3 and 4 and decreased at pH 9 and 10 (see Appendix).

Shoot number in cytokinin treatments and root number in auxin treatments were recorded after a four week culture period. Callus formation was also noted.

Results

Neither of the species tested survived at pH 3 or 10, and Crataegus did not survive at pH 8 or 9. Shoots of Rhododendron at pH 7, 8 and 9, and shoots of Crataegus at pH 7, were short and chlorotic.

Shoot formation

Shoot number is given in Tables 215 and 216. An analysis of variance 'Table 217) showed a significant effect on shoot number due to pH ($p<.001$), to cytokinin concentration ($p<.001$) and a significant difference ($p<.001$) between species. A significant interaction ($p<.001$) between these was also demonstrated. In

Crataegus, the greatest number of shoots was formed at pH 6 and in Rhododendron at pH 5.

Root formation

Root number was greatest at lower pH than that which promoted maximal shoot number (Tables 218 and 219). An analysis of variance (Table 220) showed a significant effect on root formation due to pH ($p<.001$) and to IBA concentration ($p<.001$) but no significant difference between species. Roots were short at low pH and long and thin at high pH.

Callus formation

Callus formation occurred to a greater extent when the pH was low (Tables 215, 216, 218, 219).

Table 215. Number of shoots after 4 weeks incubation on medium with varying hydrogen ion concentration in Crataegus.

pH of medium	BA concentration mg l^{-1}			
	0	0.5	1.0	2.5
3	0	0	0	0
4	0	0	0	1.25
5	0.5	3.25	4.25	2.5
6	1.25	4.25	7.5	5.25
7	0.75	2.25	2.75	0.25
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0

Table 216. Number of shoots after 4 weeks incubation on medium with varying hydrogen ion concentration in Rhododendron 'P.J.M. Victor'.

pH of medium	BA concentration mg l^{-1}			
	0	0.5	1.0	2.5
3	0	0	0	0
4	0.25	0.25	1.25	2.5
5	0.5	2.25	4.25	3.25
6	0.25	1.75	1.0	0
7	0	1.0	0	0
8	0	1.5	0	0
9	0	0.25	0	0
10	0	0	0	0

Table 217.

Analysis of variance for data given in Tables 215 and 216.

SOURCE	S.S.	D.F.	M.S.	F	p
pH	287.547	7	41.078	164.313	<.001
BA	42.078	3	14.026	56.104	<.001
Species	16.000	1	16.000	64.000	<.001
pH * BA	99.609	21	4.743	18.973	<.001
pH * species	118.563	7	16.938	67.750	<.001
BA * species	5.969	3	1.990	7.958	<.001
pH * BA * species	46.719	21	2.225	22.583	<.001
Error	48.000	192	0.250		
Total	664.484	255			

Table 218. Number of roots after 4 weeks incubation on medium with varying hydrogen ion concentration in Crataegus.

pH of medium	IBA concentration mg l^{-1}				
	0	1.0	2.5	5.0	10.0
3	0	0	0	0	0
4	0	0	1.25	3.0	0
5	0	1.0	4.5	4.75	5.25
6	0	1.5	0.5	2.5	0.5
7	0	0	0	0	0
8	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	0	0

Table 219. Number of roots after 4 weeks incubation on medium with varying hydrogen ion concentration in Rhododendron 'P.J.M. Victor'.

pH of medium	IBA concentration mg l^{-1}				
	0	1.0	2.5	5.0	10.0
3	0	0	0	0	0
4	0.5	1.5	4.0	6.5	2.0
5	0	0	3.5	4.0	2.5
6	0	0	0	0.25	0
7	0	0	0	0	0
8	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	0	0

Table 220.

Analysis of variance for data given in Tables 218 and 219.

SOURCE	S.S.	D.F.	M.S.	F	p
pH	233.038	7	32.291	112.533	<.001
IBA	64.206	4	16.052	54.259	<.001
Species	0.200	1	0.200	0.676	N.S.
pH * IBA	164.744	28	5.884	19.889	<.001
pH * species	73.350	7	10.479	35.421	<.001
IBA * species	1.081	4	0.270	0.913	N.S.
pH * IBA * species	34.869	28	1.245	8.855	<.001
Error	71.000	240	0.2958		
Total	642.488	319			

6.4 DISCUSSION

Sucrose

Formation of shoots, roots and callus did not occur in the absence of sucrose. Other carbohydrates could possibly substitute for sucrose but this was not tested here. Other workers have shown an exogenous carbohydrate supply to be essential for sustained growth of callus cultures (Street, 1973), xylogenesis in cultured plant tissues (Fosket and Roberts, 1964) and root formation in stem explants of Rhododendron Pierik and Steegmans, 1975). However, whereas sucrose was required for shoot growth from buds, omission of sucrose stimulated callus formation from bud explants in Citrus (Giladi et al., 1977). This apparent anomaly may be explained by the fact that buds normally have a high endogenous sugar content, whereas explants used by other workers (e.g. stems) have a lower endogenous sugar content. Buds promote cytodifferentiation and sucrose combined with IAA could substitute for buds in promoting cytodifferentiation (Wetmore and Rier, 1963).

The shoot explants used for my experiments contained chlorophyll and therefore might be expected

to be capable of carbohydrate synthesis. However, an additional carbohydrate source was essential for differentiation or dedifferentiation to occur. This may be because leaves were removed from the explants and so photosynthesis was reduced to a level insufficient to provide energy for much growth. Tran Thanh Van et al. (1974) showed that vegetative buds in thin cell layers of Nicotiana were formed in 11 days in the presence of sucrose, and 30 days later in the absence of sucrose. Myo-inositol, incorporated in most tissue culture media including those used in the experiments described in this thesis, can act as a carbon source for cytodifferentiation (Roberts and Baba, 1982), and could be utilized in the absence of sucrose. Pretreatment of lettuce pith explants with sucrose prior to culturing them on a xylogenic medium, shortened the lag phase of xylem differentiation (Cawthon, 1972). In my experiments, shoot, root and callus formation was recorded after 28 days. It is possible, based on the above literature, that organogenesis might have occurred without exogenous sucrose if a longer incubation time had been given.

Sucrose concentration had a marked effect on differentiation. Concentrations of 20 to 40 g l-1

were optimal for callus, shoot and root formation. There was no difference in sucrose requirement for differentiation and dedifferentiation, and sucrose concentration did not determine direction of differentiation. This is contrary to a previous report for tobacco (Tran Thanh Van, 1977) in which it was shown that optimal concentrations were 30 g l^{-1} for vegetative bud formation, 10 g l^{-1} for root formation and 30 g l^{-1} for callus growth.

At high sucrose concentrations, red pigmentation was observed in shoots. Other workers have shown that high levels of sucrose can markedly inhibit chloroplast differentiation (Davey et al., 1971) and promote ethene biosynthesis (Moore, 1976). This could lead to senescence and the red colouration observed. Reduction of shoot, root and callus formation also occurred at high sucrose concentrations. This could be an indirect effect via ethene.

Ball (1953) reported that autoclaving a 3% sucrose medium resulted in a medium containing from 0.7 to 0.9% of a mixture of D-glucose and D-fructose in addition to sucrose. The beneficial effect observed on differentiation and callus growth therefore could be due to these sugars rather than to sucrose. Romberger

and Tabor (1971) found that autoclaving sucrose, in the presence of agar, improved the growth of shoot apical meristems, and Ball (1953) showed that callus of Sequoia grown on culture media containing either autoclaved or filter-sterilized sucrose, produced two distinctly different types of callus growth, autoclaved sucrose giving enhanced callus growth. However, Stehsel and Caplin (1969) found that filter sterilization gave more growth in carrot tissue.

Sucrose may control differentiation and dedifferentiation by (1) supplying energy for growth, 2) interacting with growth regulators, or 3) by changing the osmotic potential of the medium.

Increasing the concentration of carbohydrates in the nutrient medium lowers the availability of water to cultured tissues, resulting in water stress which may influence cytodifferentiation (Roberts, 1976). Doley and Leyton (1970) found greater production of xylem elements in callus of Fraxinus after the water potential of the medium had been lowered with either sucrose or PEG; and cell division , as measured by DNA increase, was stimulated by increased turgor pressure (Kirkham et al., 1972).

However, neither osmotic effects nor its role as

an energy source account for all of the activity of sucrose in differentiation and growth. Structure of the sucrose molecule, specifically the α -glucosyl residue at the non-reducing end of the molecule, is important in its activity, implying an interaction with a specific binding site (Hall, 1976); and Cawthon (1972) showed that greater numbers of tracheary elements were formed with sucrose than with the combination of the two monosaccharides D-glucose and D-fructose, demonstrating the importance of disaccharide structure in morphogenesis. Others have shown that sucrose has a growth-regulator-like effect on differentiation, especially in combination with auxin. Wetmore and Rier (1963) found that anatomy of vascularized nodules in callus was changed by increasing exogenous sucrose concentration; callus treated initially with sucrose, formed only tracheary elements, whereas callus treated initially with IAA, followed by sucrose, formed vascular nodules containing xylem, phloem and a cambium (Jeffs and Northcote, 1967); and sugar balance can reverse the effects of auxin / cytokinin ratio (Chlyah et al., 1975).

Sucrose can be degraded by sucrose synthetase to form NDP-glucose (Delmer and Albersheim, 1970), which

can directly enter the reactions producing primary and secondary wall monomers, whereas the assimilation of either D-glucose or D-fructose requires the expenditure of ATP energy for phosphorylation, and these sugar phosphates would be more readily available as substrates for the glycolytic pathway than for wall metabolism (Roberts, 1976). This partially explains the difference in activity between mono- and disaccharides.

Occlusion of organic substances, particularly traces of amino acids, occurs during crystallization of sucrose (Schneider et al., 1975); and Torrey et al. (1971) suggested that sucrose could be contaminated with traces of naturally occurring plant hormones such as cytokinins. These contaminants may also affect differentiation.

Mineral content

All of the potassium salts affected shoot, root and callus formation, whereas the other salts had little effect. Shoot and callus formation was decreased by omission of potassium salts, whereas root formation was increased. This indicates that potassium may be important in determining direction of

differentiation. Elimination of magnesium salts also increased rooting but had no effect on shoot or callus formation.

Other workers have shown that a high salt concentration decreases rooting in vitro (Lane, 1978 : apple) and KI inhibited rooting in Ericaceous and Rosaceous species (Anderson, 1981). Potassium is detrimental to survival of shoot explants (Anderson, 1975), and can decrease root formation (Khalighy, 1976 , in Dianthus; Joiner and Gruis, 1966; Lovell et al., 1971).

Increased levels of a rooting cofactor (cofactor 4) in cuttings of Dianthus were reported when stock plants were treated with fertilizer without potassium (Khalighy, 1976) in other species, this could explain the observed increase in root formation in my experiments when potassium was omitted from the medium (see Chapter 4 for discussion of the role of cofactors in rooting).

Other changes which may affect plant growth have also been reported when plant tissues are cultured on nutrient medium without potassium. Cohen (1968) recorded increased absorption of nitrates in the absence of potassium in cultured carrot tissue. Cohen also noted that synthesis of proteins was decreased,

proportions of free-amino acids were changed, absorption of glucose and synthesis of sucrose were decreased, and content of energy rich phosphate compounds decreased. Several of these changes involve a decrease in energy. Potassium is bound to pyruvate kinase which is essential in respiration and carbohydrate metabolism (Bidwell, 1979) and thus, a reduction in potassium concentration can reduce the energy available for differentiation. Protein synthesis necessary for differentiation can also be controlled by potassium which is essential for activation of the enzymes involved (Bidwell, 1979).

BA treatment stimulated potassium uptake in peach seedlings (Richards, 1978) - this could reflect an increase in demand brought about by increased growth. Long-term uptake of nutrients is determined mainly by metabolic demand (Russell, 1972; 1977). Morel (1975) noted that a high concentration of potassium (as provided in Murashige and Skoog 1962 medium) is required for growth and development of cultured shoot apices; and Heller (1965) showed that the requirement for potassium by cultured tissues increases as intensity of tissue proliferation increases, but noted that the relative requirements for calcium decrease

under those circumstances. Calcium has been shown to inhibit the hydrogen ion induced cell wall loosening process (Cleland and Rayle, 1977) and thus can inhibit growth, but calcium elimination did not increase root or shoot initiation in the current experiments.

Part of the effect of potassium could be due to the change in balance of monovalent to divalent ions in the medium brought about by elimination of potassium. This could affect the ionic balance of the cells and thus lead to uptake or discharge of other ions to balance the charge. Such changes could affect the mineral balance of the plant tissue or the internal or external hydrogen ion concentration. It is proposed that additional hydrogen ions may be absorbed in place of potassium ions, thus leading to a decreased pH in the cells. A low pH favours root formation but not shoot formation (see below). This could explain the observed increase in root formation and decrease in shoot formation when potassium was omitted from the medium.

The results of this experiment are not absolutely conclusive as entire salts were excluded from the medium. Therefore, the observed effects could be due to either the positive or the negative part of the

molecule. However, the fact that all of the potassium salts affected differentiation whereas other salts had a much lesser effect strongly indicates that the observed effect is due to potassium. **

Hydrogen ion concentration

Shoot formation was promoted at a higher pH than was root or callus formation. This may demonstrate that hydrogen ion concentration is important in determining direction of differentiation. Others have also shown that an acid medium favours rooting (Hartmann and Kester, 1975). Increasing the external hydrogen ion concentration results in cell wall loosening (Rayle and Cleland, 1977) with resultant cell expansion. Auxin promotes cell expansion in the same way and therefore, high hydrogen ion concentration may enhance auxin action. However, cell expansion is involved in both root and shoot formation and this does not explain why a different pH is required for these two types of differentiation.

Suspension cultures of rose required a low pH (5.2-5.4) during the division phase and a higher pH (5.8-6.0) during the expansion phase (Nesius and Fletcher, 1973). However, cell division and expansion

**Elimination of other salts had no effect on differentiation. This may indicate that other minerals are stored in the explants for four weeks (the incubation period in this experiment) or longer, whereas potassium is depleted more rapidly.

are involved in differentiation.

It appears, therefore, that differentiation may not be controlled by hydrogen ion exchange with plant cells. Marre (1977) suggested that differentiation is due to effects other than the activation of H^+/K^+ exchange.

It is difficult to separate the effects of pH per se from pH-induced effects on nutrient availability. Nitrogen uptake and metabolism are affected by pH (Adreenko and Alekhina, 1966, 1967) and nitrogen level can affect endogenous cytokinin level (Salama and Wareing, 1979). Hydrogen ion concentration could therefore indirectly affect growth regulator balance and, in this way, affect the pattern of differentiation.

VII. RESPIRATION IN SHOOTS CULTURED IN VITRO.

7.1 RESPIRATION RATE AFTER AUXIN AND CYTOKININ TREATMENT

A decline in shoot and plant vigour with propagative generation was recorded and was paralleled by a decline in root initiation (Chapters 3 and 4). It is hypothesised that a change in energy production (from sucrose) occurs and is partially responsible for this decline. In Chaenomeles, which forms axillary shoots only, a decline in rhizogen ic potential with propagative generation was observed. An experiment was conducted to determine whether a change in respiration occurs in adventitious shoots (Spiraea) or axillary shoots (Chaenomeles) with propagative generation.

Respiration rate has been shown to both increase and decrease after auxin or cytokinin application (Bonner,1933; Moore and Miller, 1972; Humphreys and Dugger, 1957; Tetley and Thimann, 1974). This may indicate that change in respiration rate is a result of growth regulator induced growth rather than a direct result of growth regulator application. Respiration rate was examined at different auxin and cytokinin concentrations in relation to differentiation and growth to investigate this.

Method

First and second generation shoots (see Chapter 3) of Chaenomeles japonica and Spiraea 'Froebelii' were cultured on nutrient medium containimng BA or IBA at the following concentrations:-

BA - 0, 0.1, 0.5, 1.0, 2.5, 5.0 mg l^{-1}.

IBA - 0, 1.0, 2.5, 5.0, 10.0, 20.0 mg l^{-1}.

Respiration rate was assayed at the end of a two week incubation period in light (16 hour photoperiod) for BA cultures and in dark (one week) followed by light (16 hour photoperiod)(one week). Respirometry is detailed in Chapter 2.

Results

Cytokinin.

Fitted curves for change in respiration rate with BA concentration are given in Figures 423 to 426. In generation 1, respiration rate increased with increase in BA concentration, but decresed at high BA concentrations in both species.

In generation 7, the same pattern was evident for Chaenomeles, but in Spiraea, respiration rate declined with increasing BA concentration.

An analysis of variance (Table 221) showed a significant effect on respiration rate due to BA concentration ($p<.001$), a significant effect due to propagative generation ($p<.001$).

Respiration rate was less for generation 7 cultures than for generation 1 cultures, and a significant difference due to species ($p<.001$).

Auxin

An increase in respiration rate with increasing IBA concentration occured in both species (Figures 427 to 430). In generation 7, respiration rate in cultures of Spiraea increased with IBA concentration to a greater extent than in cultures of Chaenomeles.

An anlysis of variance (Table 222) showed a significant effect on respiration rate due to IBA concentration ($p<.001$), generation ($p<.001$), and species ($p<.001$).

Figures 423 to 426.

Respiration rate (oxygen consumed) after incubation on media with different BA concentrations.

Figures 427 to 430.

Respiration rate (oxygen consumed) after incubation on media with different IBA concentrations.

Fig 423. Generation 1. C. japonica.

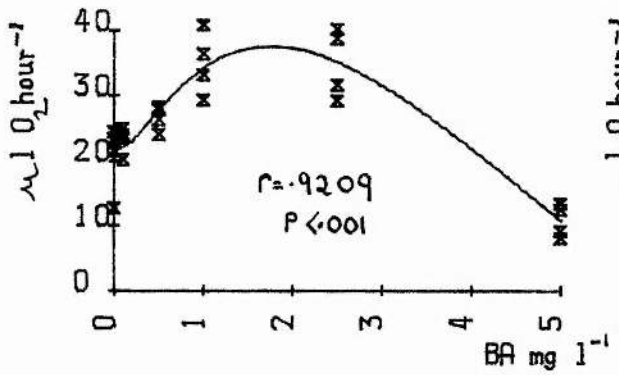


Fig 424. Generation 7. C. japonica.

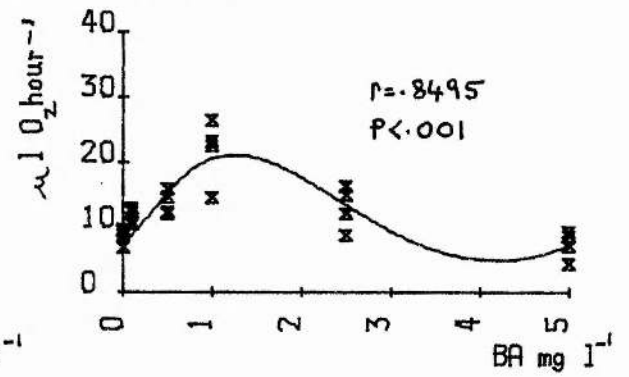
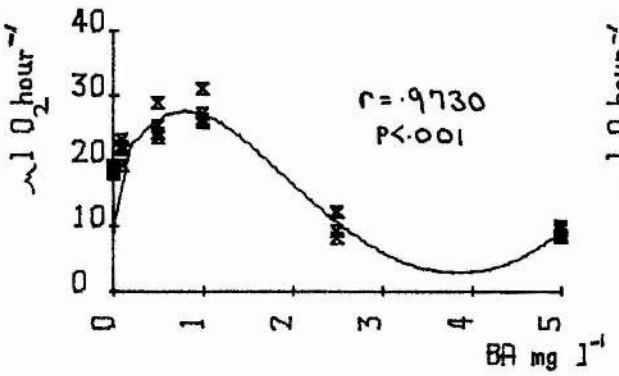


Fig 425. Generation 1. S. 'Froebelii'.



Generation 7.

Fig 426 S. 'Froebelii'.

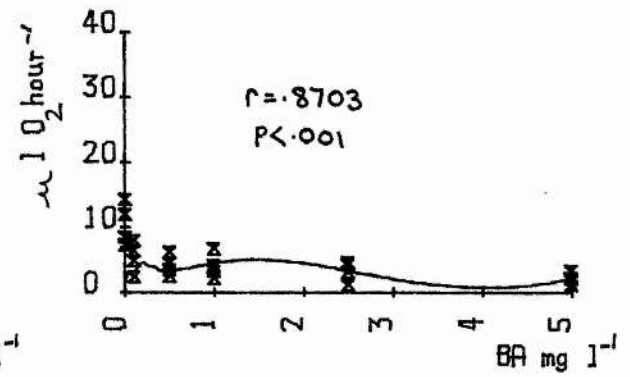


Fig 427. Generation 1.C. japonica.

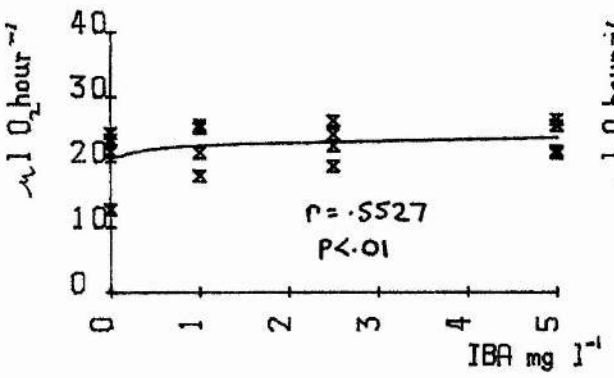


Fig 428. Generation 7.C. japonica.

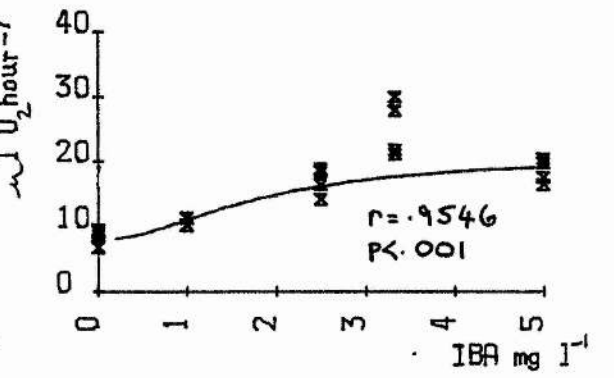
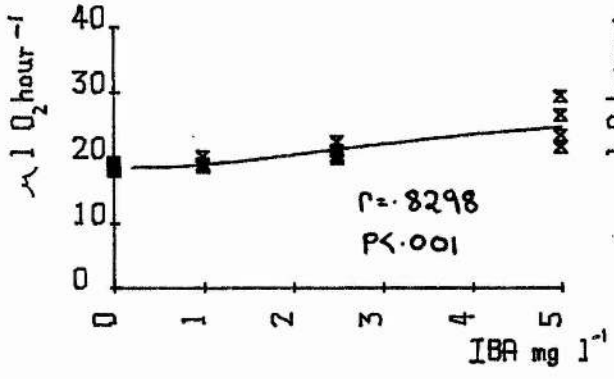


Fig 429. Generation 1.S. 'Froebelii'.



Generation 7.

Fig 430 S. 'Froebelii'.

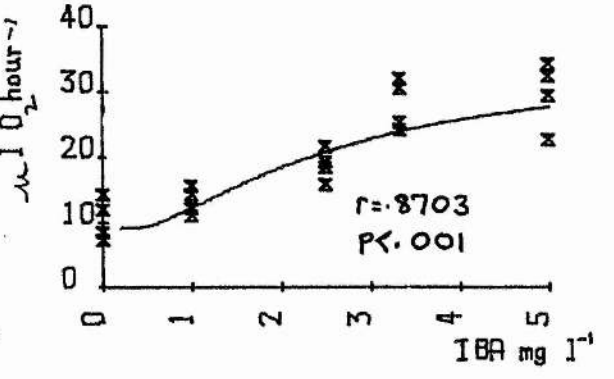


Table 221.

Analysis of variance for data given in Figures 423 to 426.

SOURCE	S.S.	D.F.	M.S.	F	p
BA concentration	1826.538	5	365.308	46.559	<.001
Generation	4187.834	1	4187.834	533.751	<.001
Species	1168.923	1	1168.923	148.983	<.001
BA * generation	478.984	5	95.797	12.210	<.001
BA * species	828.114	5	165.623	21.109	<.001
Generation * species	11.609	1	11.609	1.480	N.S.
BA * generation * species	430.598	5	86.120	21.109	<.001
Error	564.916	72	7.8460		
Total	9497.521	95			

Table 222.

Analysis of variance for data given in Figures 427 to 430.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA concentration	2460.740	5	492.148	30.103	<.001
Generation	344.791	1	344.791	21.090	<.001
Species	317.481	1	317.481	19.419	<.001
IBA * generation	455.635	5	91.127	5.574	<.001
IBA * species	369.867	5	73.973	4.525	<.01
Generation * species	385.169	1	385.169	23.560	<.001
IBA * generation * species	113.142	5	22.628	4.525	<.01
Error	1177.102	72	16.349		
Total	5623.928	95			

7.2 DISCUSSION

Cytokinin and respiration rate

In the first propagative generation, the same pattern of respiration rate with concentration was evident in both species examined. Respiration rate rose with concentration, but declined at high BA concentrations.

Others have reported a stimulation of respiration by cytokinin in cultured tissues, for example, in tobacco pith (Glaszion, 1957) and soybean callus (Moore and Miller, 1972), and at high cytokinin concentrations, respiration was inhibited in Acer (Simpkins and Street, 1970) and soybean callus (Moore and Miller, 1972). My work supports these findings.

Promotion and inhibition of respiration rate with BA concentration paralleled the curve for shoot formation with concentration (see Chapter 3). As respiration rate increased, shoot initiation increased. A correlation of respiration rate with shoot formation was also reported by Thorpe and Meier (1972). This may show that (1) an increase in respiration rate is necessary before shoot initiation can take place, (2) the increase in respiration rate is a result of

increased growth brought about by shoot formation, or (3) increase in respiration rate is parallel to but unrelated to shoot formation.

In generation 7, respiration rate was lower than in generation 1 in both species. Repeated cytokinin treatment could have caused a decline similar to that which occurred at high BA concentrations in generation 1. However, a difference in respiration rate with BA concentration between Spiraea and Chaenomeles was shown. Whereas, in Chaenomeles, the same pattern of rise and fall with concentration as in generation 1 occurred, in Spiraea, a decline in respiration rate with increasing BA concentration was noted. This suggests that differentiation and respiration are linked, as explants of generation 7 (Spiraea) did not respond to BA by initiating as many shoots as in generation 1, whereas this decrease was not as marked in Chaenomeles.

Auxin and respiration

An increase in respiration rate with increase in IBA concentration was recorded for both species in generations 1 and 7. Auxin increases respiration rate in other species, for example, Avena (Bonner, 1933),

but high auxin concentrations have been shown by others to inhibit respiration (Humphreys and Dugger, 1957).

Change in respiration rate with IBA concentration did not parallel differentiation as was the case for cytokinin. An increase in respiration rate normally accompanies an increase in growth (Thimann, 1977), and an increased energy supply is necessary to sustain increased growth. Respiration rate in non-growing tissues can increase after auxin treatment (Michel, 1951), while in other tissues, auxins can inhibit respiration but stimulate growth (Marinos, 1957). This indicates that growth and respiration are not necessarily direct consequences of each other. When growth and respiration both increased in response to auxin, Rowan et al. (1972) showed that the stimulation of respiration occurs after auxin-induced growth has commenced. Therefore, growth regulator-stimulated changes in growth and differentiation are unlikely to be caused by a change in respiration rate.

In generation 7, respiration rate increased with IBA concentration to a greater extent in Spiraea than in Chaenomeles, such that the rate at high IBA concentrations was greater than that recorded in generation 1 (in Spiraea). In this case, there is no

correlation of differentiation with respiration rate, as no roots formed at these concentrations. Increased energy resulting from increased respiration in auxin-treated shoots was apparently not utilized for growth, for example, for cell wall building. The energy may therefore be diverted to other activity, for example, mineral uptake. Increased mineral uptake could result in the observed decrease in root formation (see Chapter 6).

VIII. THE ROLE OF ETHENE IN MORPHOGENESIS.

VIII. The role of ethene in morphogenesis

This section addresses the following questions:-

1. Does ethene promote morphogenesis in shoot explants of Rosaceous plants?
2. What effect does ethene concentration have on morphogenesis?
3. Is ethene a mediating compound regulating morphogenetic response to auxin and cytokinin?
4. Does ethene synthesis change in relation to exogenous auxin and cytokinin concentration?
5. Is there a correlation between ethene synthesis and morphogenesis?

To determine the effect of ethene on morphogenesis, ethene was supplied to shoot explants either as gas or as the ethene-releasing compound 2-chloroethylphosphonic acid (ethephon).

The effect of auxin and cytokinin concentration on ethene synthesis was assessed by measuring ethene in culture vessels after auxin and cytokinin treatment.

Change in sensitivity of the explant (in terms of ethene synthesis) to auxin and cytokinin was determined by assaying ethene over a four week time period.

8.1 ETHENE AND 2-CHLOROETHYLPHOSPHONIC ACID ACTIVITY IN MORPHOGENESIS

Method

Shoot explants of Prunus cerasifera and Spiraea 'Froebelii' were used. Ethene was added to cultures in one of the following ways:-

1) Ethene gas injected to culture tubes to give an initial concentration of 10ppm.

2) Ethephon at the following concentrations :- 0, 1, 5, 10, 50 or 100 mg l-1 added to the nutrient medium a) prior to autoclaving or b) after autoclaving.

3) No ethene or ethephon supplied.

4) BA or IBA addition to the medium at concentrations previously demonstrated to promote the formation of shoots or roots.

Shoot, root and callus formation were recorded at the end of a four week incubation period in light (16 hour photoperiod).

Results

Shoot formation

Polynomial curves were fitted for change in shoot number with concentration of ethephon for Prunus (Figure 431) and for Spiraea (Figure 432). The fit of the curves was significant at the 99.9% level. From these curves, optimal shoot numbers and concentrations of ethephon were calculated. These are given below.

Prunus	Spiraea	
3.29 (7ppm)	12.23 (10ppm)	autoclaved
1.84 (3ppm)	6.34 (19ppm)	non-autoclaved

Analysis of variance (Table 224) showed a significant effect due to treatment ($p<.001$) and a significant difference between species ($p<.001$). Table 223 shows that the order of treatments in terms of shoot number was (1) BA with most shoots, (2) ethephon (autoclaved), (3) ethene and non-autoclaved ethephon, (4) control with least shoots. Groups (1) to (4) were all significantly different ($p<.05$). Spiraea produced consistently more shoots than Prunus.

At the concentration tested, ethene gas promoted

shoot formation in Spiraea but not in Prunus (Table 223).

Shoot number in ethene and ethephon treatments was significantly less than that for cytokinin-treated explants (Table 223).

Root formation

Polynomial curves were fitted for change in root number with ethephon concentration (Figures 433 and 434). Fit of curves was significant at the 99.9% level. From these curves, optimal root numbers and optimal ethephon concentrations were calculated and these are given below.

Prunus	Spiraea	
9.22 (5ppm)	12.54 (11ppm)	autoclaved
3.61 (0.3ppm)	2.40 (0.4ppm)	non-autoclaved

An analysis of variance (Table 226) showed a significant effect due to treatment ($p<.001$) and a significant difference between species ($p<.001$). Ethephon promoted rooting in both Prunus and Spiraea. Root number varied with concentration (Figures 426 to 427). A higher concentration of ethephon was required to promote root formation when

ethephon was autoclaved. Ethene gas also promoted root formation (Table 225). The order of effectiveness of treatments was 1. IBA, ethephon (autoclaved), 2. ethene, ethephon (not autoclaved), 3. control with fewest roots ($p<.05$).

A significantly greater number of roots formed in Spiraea than in Prunus ($p<.001$). There was a significant interaction between species and treatment ($p<.001$), whereas IBA gave most roots in Spiraea, ethephon gave most roots in Prunus (Table 226).

Callus formation

Callus was not formed in ethephon or ethene treatments.

Other observations

Red pigmentation in leaves of Prunus was observed when autoclaved ethephon at 100 mg l^{-1} or non-autoclaved ethephon at 5 and 10 mg l^{-1} was supplied. Senescence was observed when non-autoclaved ethephon was given at 50 and 100 mg l^{-1}.

Figures 431 and 432.
Mean shoot number after incubation on medium containing ethephon (autoclaved or non-autoclaved).

Figures 433 and 434.
Mean root number after incubation for four weeks on medium containing ethephon.

Fig 43. Prunus cerasifera.

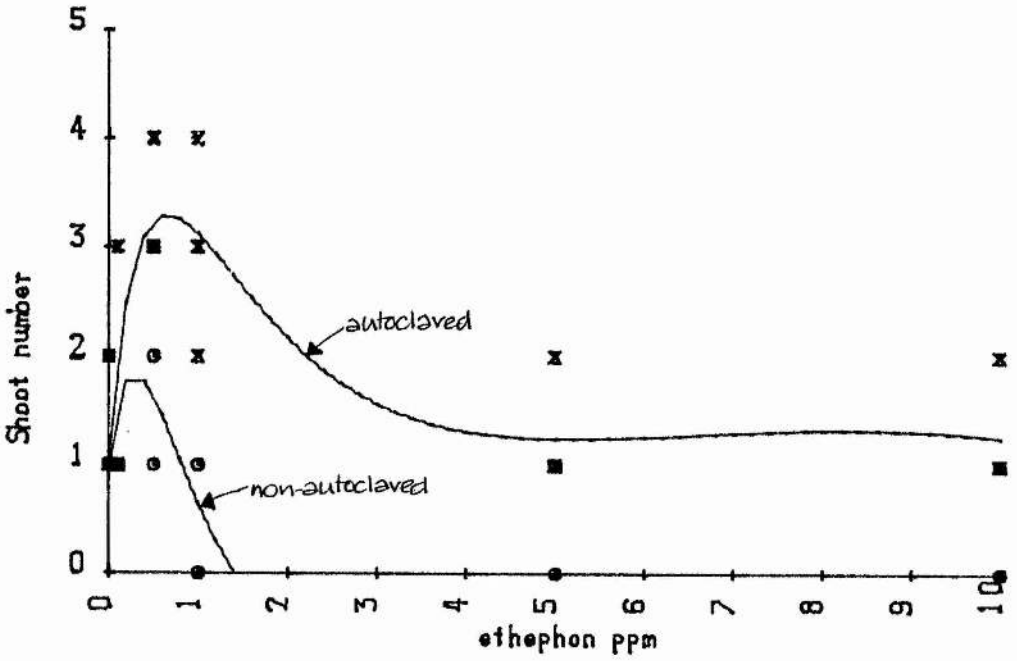


Fig 432 Spiraea 'Froebelii'.

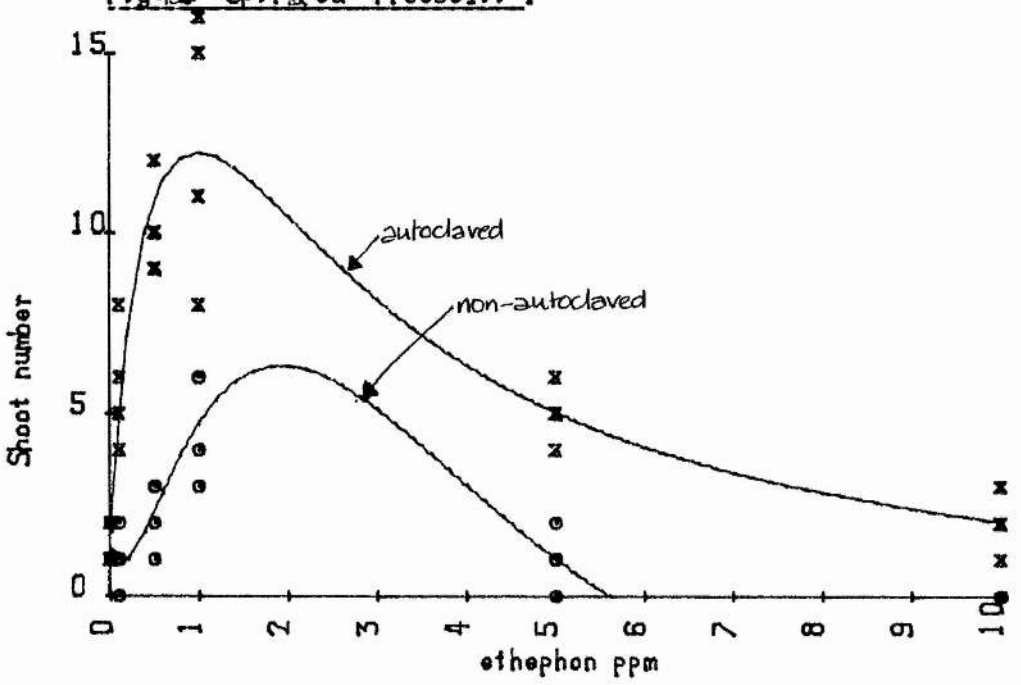


Fig 433 Prunus cerasifera.

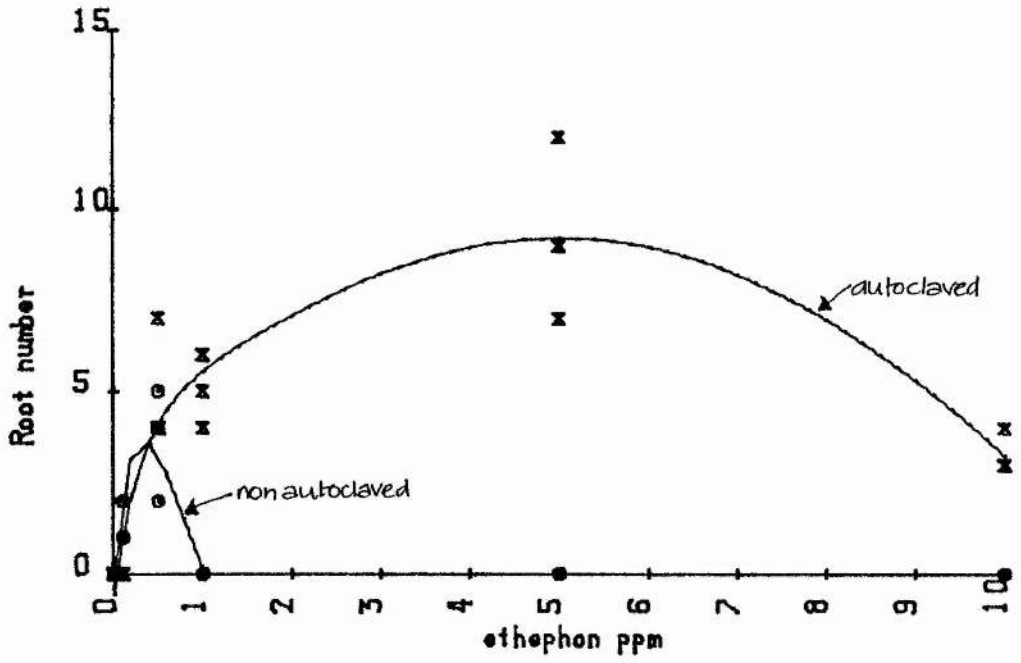


Fig 434. Spiraea 'Froebelii'.

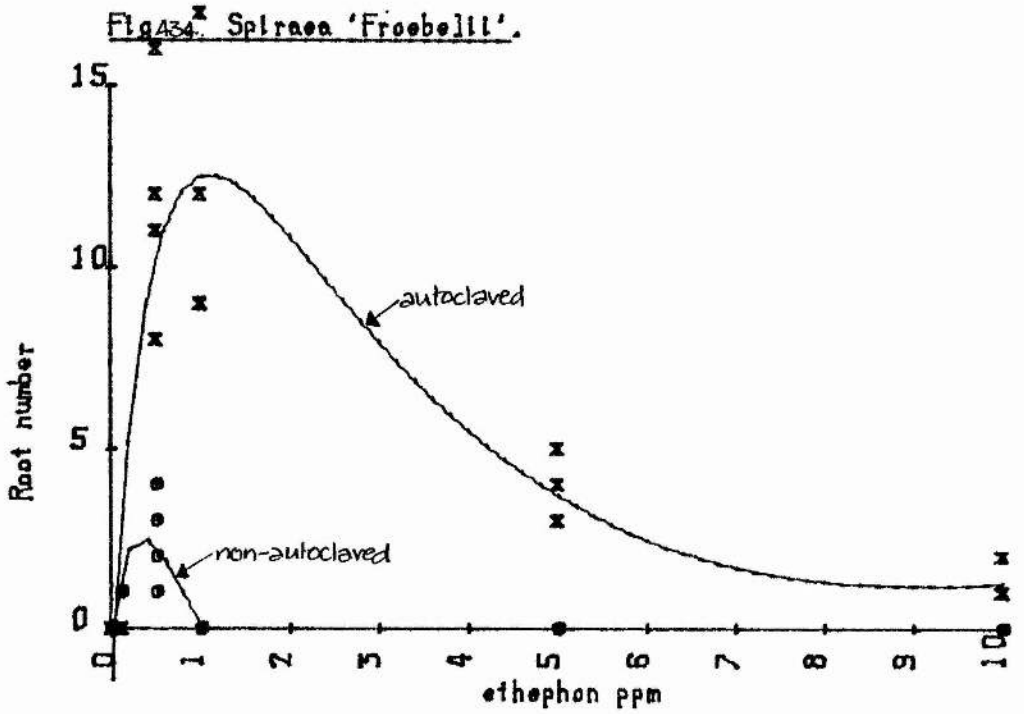


Table 223.

Mean shoot number after ethephon and ethene treatment.

treatment	Prunus	Spiraea	mean
control	1.25	1.25	1.25d
BA	5.0	28.0	16.5a
ethene	1.75	5.5	3.625c
ethephon(a)	3.5	12.5	8.0b
ethephon(na)	2.0	4.8	3.38c
Mean	2.7b	10.4a	

Means followed by different letters are significantly different ($p<.05$).

Table 224.

Analysis of variance for data given in Table 223.

SOURCE	S.S.	D.F.	M.S.	F	p
treatment	1182.65	4	295.66	88.69	<.001
species	592.90	1	592.90	177.87	<.001
Interaction	670.35	4	167.59	50.28	<.001
Error	100	30	3.3333		
Total	2545.9	39			

Table 225.

Mean root number after ethephon and ethene treatment.

treatment	Prunus	Spiraea	mean
control	0	0	0c
IBA	1.50	20.00	10.75a
ethene	1.75	5.25	3.50b
ethephon(a)	9.25	11.75	10.50a
ethephon(na)	3.75	2.50	3.13b
Mean	3.25b	7.90a	

Means followed by different letters are significantly different ($p<.05$).

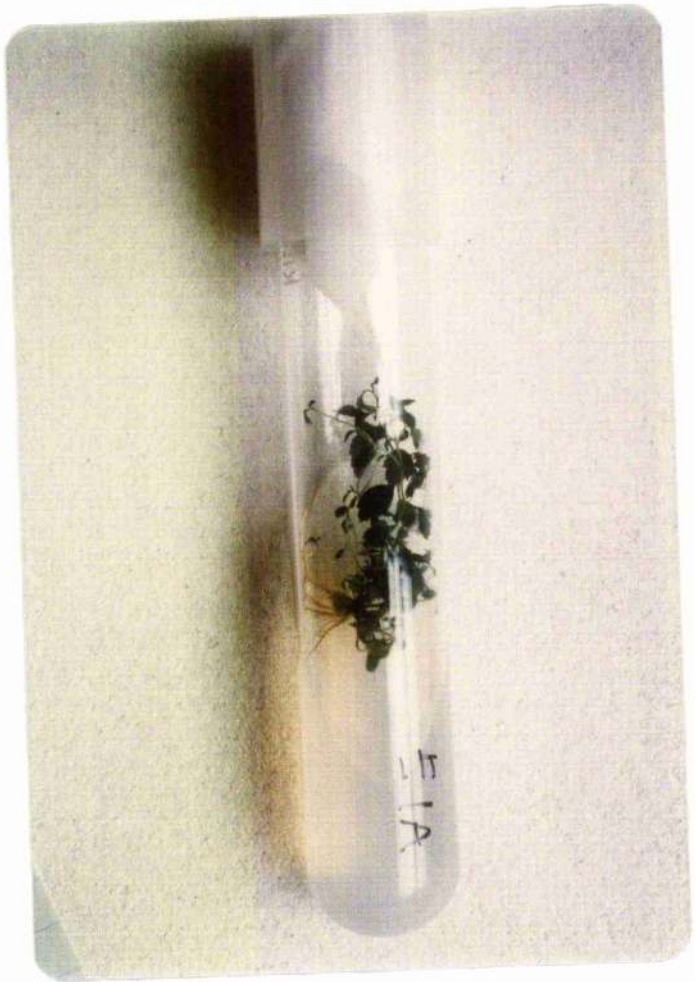
Table 226.

Analysis of variance for data given in Table 225.

SOURCE	S.S.	D.F.	M.S.	F	p
treatment	739.40	4	184.85	57.92	<.001
species	216.23	1	216.23	67.75	<.001
Interaction	508.40	4	127.10	39.82	<.001
Error	95.75	30	3.1917		
Total	1559.78	39			

Plate 20.

Spiraea 'Froebelii' after ethephon treatment - simultaneous shoot and root formation.



8.2 ETHENE SYNTHESIS AFTER AUXIN AND CYTOKININ TREATMENT

Method

Experiment 1.

Shoot explants of the following species were used in the experiment :- *Chaenomeles japonica*, *Cotoneaster dammeri*, *Crataegus brachyacantha*, *Potentilla fruticosa* 'Coronation Triumph', *Prunus cerasifera*. IBA or BA was incorporated in the nutrient medium at the following concentrations:-

IBA - 0, 1.0, 2.5, 5.0, 10 or 20 mg l^{-1}

BA - 0, 0.1, 0.5, 1.0, 2.5 or 5.0 mg l^{-1}.

Ethene levels in the gaseous volume of the culture tube was recorded after a two week incubation period in light (16 hour photoperiod). This time was chosen because previous experiments (Chapters 3 and 4) showed shoot and root formation activity, in terms of visible morphogenesis, to be most intense after two weeks in culture. For details of ethene assay see Section 2.5.

Results were calculated in terms of (1) ppm ethene per culture, and (2) ppm per mg dry weight of tissue. Ethene in control tubes (no plant) was subtracted from

each result.

Experiment 2.

Shoot explants of Prunus cerasifera were cultured on nutrient medium containing 0.5 mg l^{-1} BA, 2.5 mg l^{-1} IBA, or no growth regulator. These concentrations were previously shown to promote shoot or root formation (Chapters 3 and 4). Ethene levels were recorded after 1, 7, 14, 21 and 28 days incubation in light (16 hour photoperiod).

Results

Experiment 1.

Polynomial curves of the form

$y = b_0 + \log_e b_1 x + \log_e b_2 x^2 + \log_e b_3 x^3 + \log_e b_4 x^4$

were plotted for ethene synthesis ($p<.001$ in each case) (Figures 435 to 444 for IBA, Figures 445 to 454 for BA). Maximal ethene synthesis and optimal BA and IBA concentrations for ethene synthesis are given in Tables 227 to 230. Ethene synthesis varied with BA and IBA concentration. Thus, at 2.5 mg l^{-1}, IBA gave more ethene than BA, whereas at 0.5 and 1.0 mg l^{-1} BA gave more ethene synthesis than IBA (Tables 231 and 232).

Tables 228 and 230 also show calculated minimum values for ethene synthesis.

An analysis of variance for ppm ethene per culture (Table 233) showed a significant effect on ethene synthesis due to BA concentration ($p<.001$), a significant effect due to species ($p<.001$), and a significant interaction between these ($p<.001$). Table 234 compares mean values for concentration and species. The order of activity in promotion of ethene synthesis for BA concentration was (1) 2.5 mg l^{-1}, (2) 1.0 and 0.5 mg l^{-1}, (3) 0.1 and 5.0 mg l^{-1}, (4) control (0 BA) ($p<.05$); and for species was (1) Chaenomeles, (2) Crataegus and Prunus, (3) Cotoneaster and Potentilla ($p<.05$).

Table 235 gives an analysis of variance for ethene per mg tissue - this shows the same effects to be significant. A comparison of means (Table 236) showed a different order of species activity in ethene synthesis.

Tables 237 and 239 give analyses of variance for ethene synthesis after IBA treatment. A significant effect due to IBA concentration ($p<.001$), species ($p<.001$) and a significant interaction between these ($p<.001$) was demonstrated.

Table 238 compares means for IBA concentrations and species (ppm ethene per culture). The order of effectiveness of IBA concentrations was (1) 20 mg l^{-1}, (2) 10 mg l^{-1}, (3) 0 IBA, (4) 5 mg l^{-1}, (5) 2.5 mg l^{-1}, (6) 1.0 mg l^{-1} ($p<.05$). The order of species was (1) Chaenomeles - produced most ethene, (2) Crataegus and Prunus, (3) Cotoneaster and Potentilla ($p<.05$). Table 240 compares means for ethene (ppm per mg). In this case, the order of effectiveness of IBA concentrations was (1) 20, (2) 10.0, (3) 5.0, (4) 2.5, (5) 1.0 mg l^{-1} ($p<.05$). The order of species in promotion of ethene synthesis was also different from that shown in Table 238.

Experiment 2.

Fitted polynomials of the form

$$y = b_0 + b_1 x + b_2 x^2 + b_3 x^3 + b_4 x^4$$

were fitted for ethene evolution against time (1 to 28 days) for BA, IBA and control cultures (Figures 455 to 457) ($p<.001$). Maximal ethene evolution was calculated from the fitted lines and is given below.

	max. ethene	days
BA	4.245	25.4
IBA	1.043	25.3
control	3.420	28

The data were subjected to an analysis of variance (Table 241). This showed a significant difference in ethene evolution with time ($p<.001$), a significant difference between treatments ($p<.001$), and a significant interaction of treatment with time ($p<.001$).

A comparison of means (Table 242) showed that BA gave the highest value for ethene evolution, and IBA gave least ($p<.05$). The order of ethene concentration with time was 28, 21, 14, 7, 1 days.

Change in ethene evolution with time was calculated, and curves of the form

$$y = b_0 + b_1 \log_e x + b_2 \log_e x^2 + b_3 \log_e x^3 + b_4 \log_e x^4$$

were fitted (Figure 458).

Table 243 gives an analysis of variance for the data. Change in rate of ethene evolution showed a change with time ($p<.001$) and treatment had a significant effect on ethene evolution ($p<.001$). There was also a significant interaction of time and

treatment ($p<.001$). The highest evolution rates were recorded at 1 day. The order of ethene evolution rate was 1, 21, 14, 7 and 28, days (Table 244). BA gave the greatest change in evolution rate.

Figures 435 to 439.
Ethene (ppm per culture) after incubation on media containing IBA.

Figures 440 to 444.
Ethene synthesis (ppm mg^{-1}) after incubation on media containing IBA.

Figures 445 to 449.
Ethene synthesis (ppm per culture) after incubation on media containing BA.

Figures 450 to 454.
Ethene (ppm mg^{-1}) after incubation on media containing BA.

Figures 455 to 457.
Ethene synthesis over a period of 28 days in cultures incubated on media containing BA, IBA or no growth regulator.

BA quartic equation	IBA	control
R^2 = .9742	R^2 = .6867	R^2 = .9461
r = .9870 (p<.001)	r = .8287 (p<.001)	r = .9727 (p<.001)
S.E. = .1942	S.E. = .1720	S.E. = .1793

Figure 458.
Change in ethene evolution over a 28 day incubation period.

Fig 435 Chaenomeles japonica.

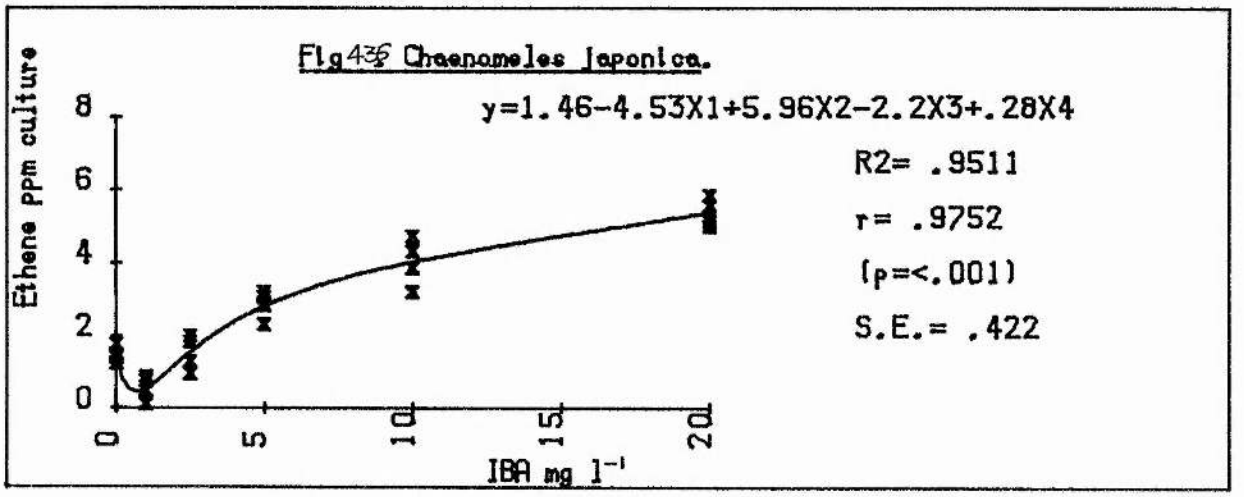


Fig 436. Cotoneaster dammeri.

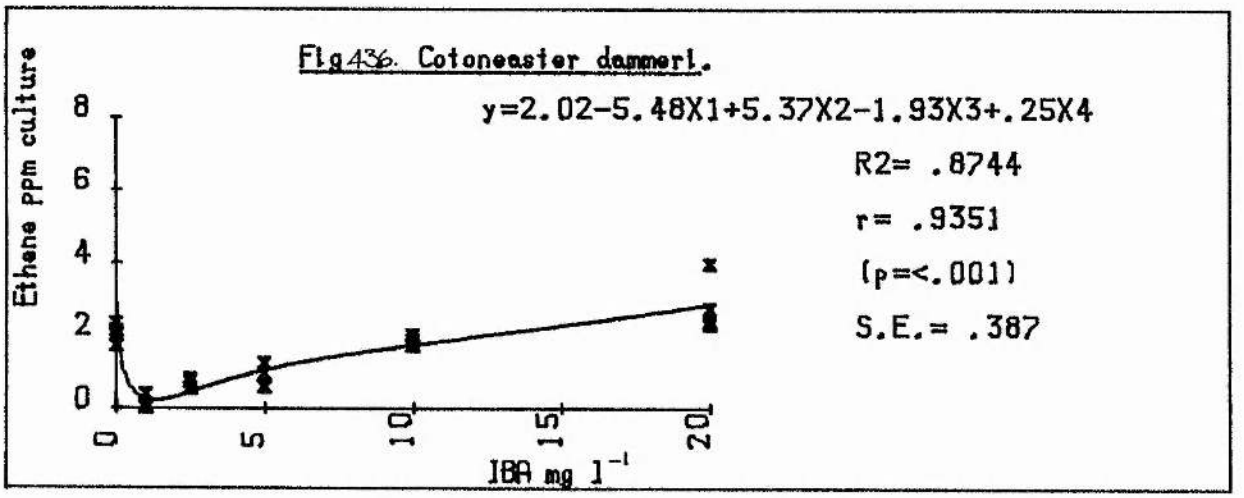


Fig 437. Crataegus brachyacantha.

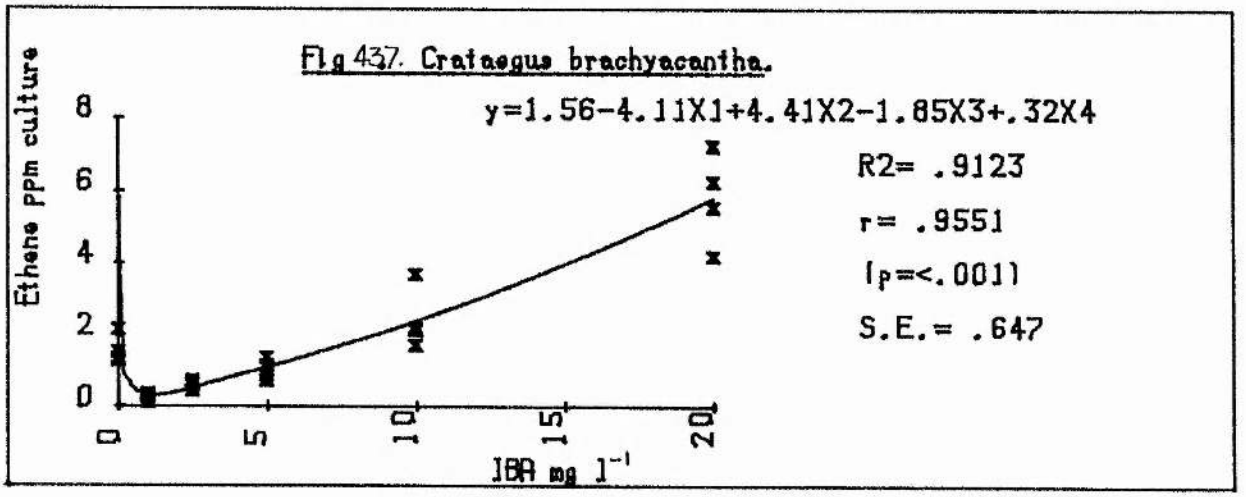


Fig 438. Potentilla 'Coronation Triumph'.

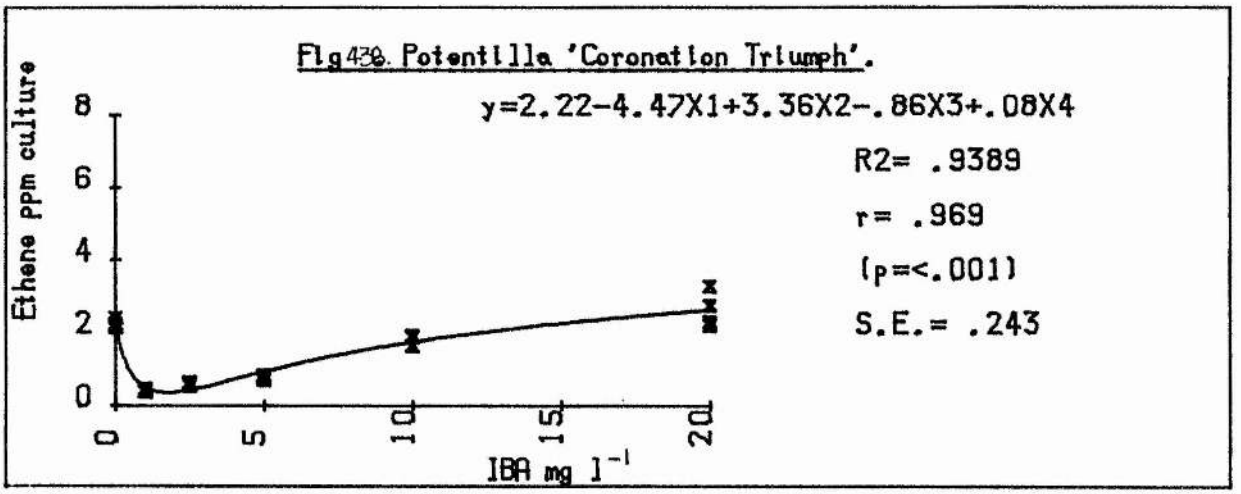


Fig 439 Prunus cerasifera.

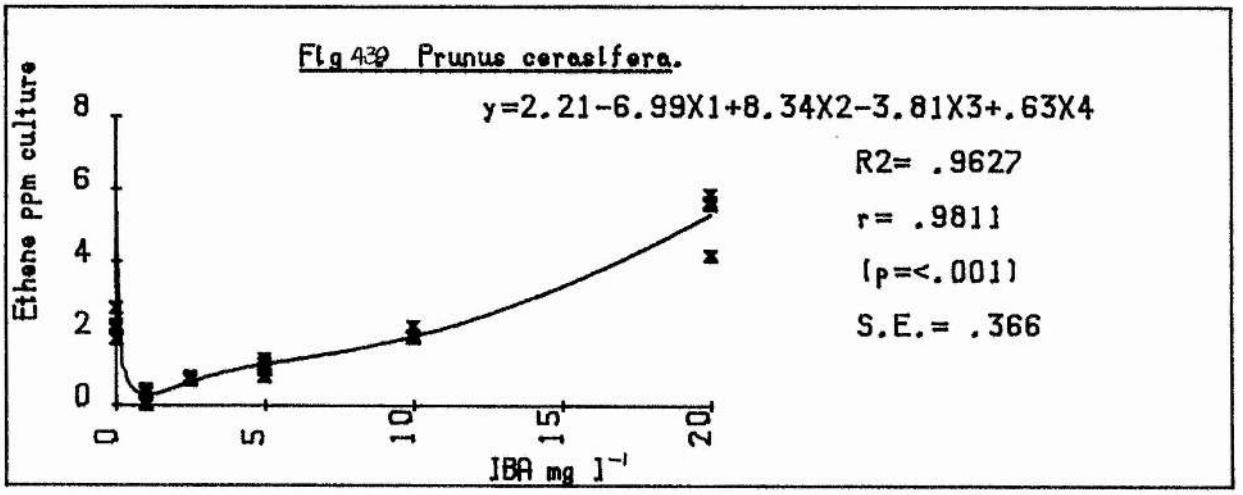


Fig 440. Chaenomeles japonica.

y=7.31-45.09X1+75.95X2-38.41X3+6.36X4

R2= .8339

r= .9131

(p=<.001)

S.E.= 5.665

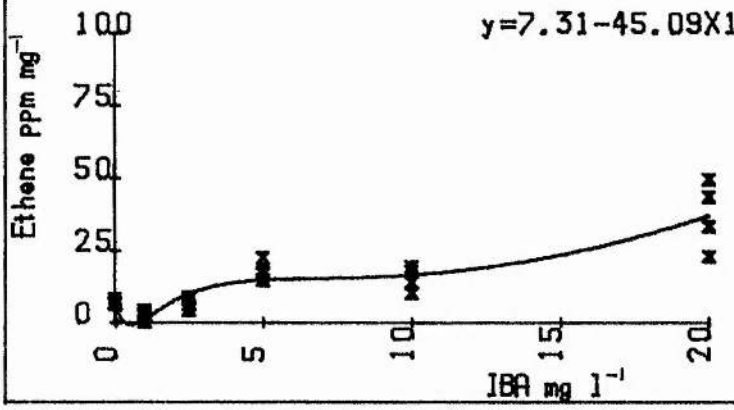


Fig 441. Cotoneaster dammeri.

y=34.65-100.43X1+110.26X2-49.07X3+8.09X4

R2= .8209

r= .906

(p=<.001)

S.E.= 10.674

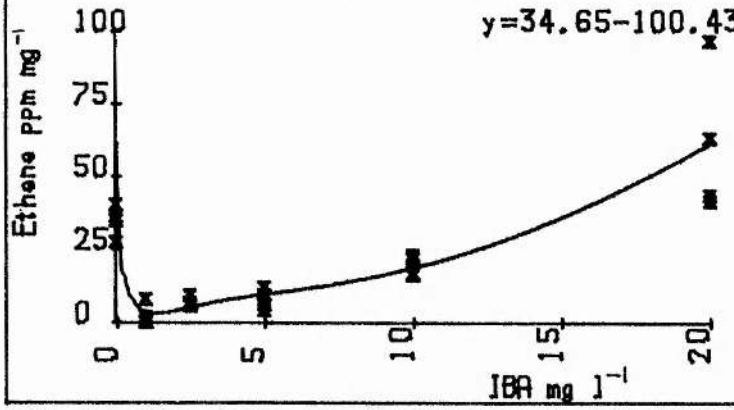


Fig 442. Crataegus brachyacantha.

y=10.05-7.24X1-9.45X2+10.98X3-1.86X4

R2= .8754

r= .9356

(p=<.001)

S.E.= 7.209

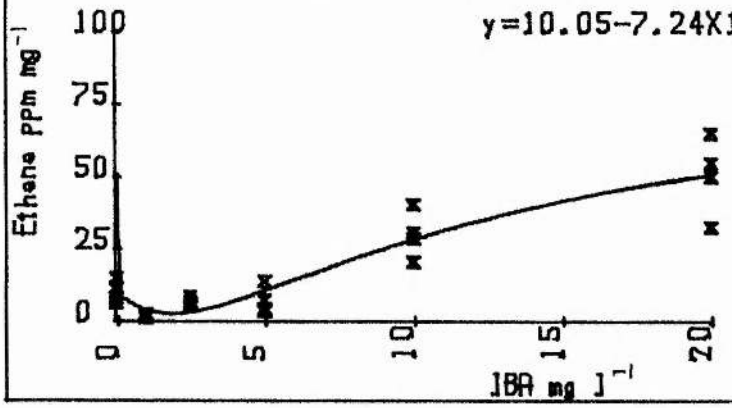


Fig 443. Potentilla 'Coronation Triumph'.

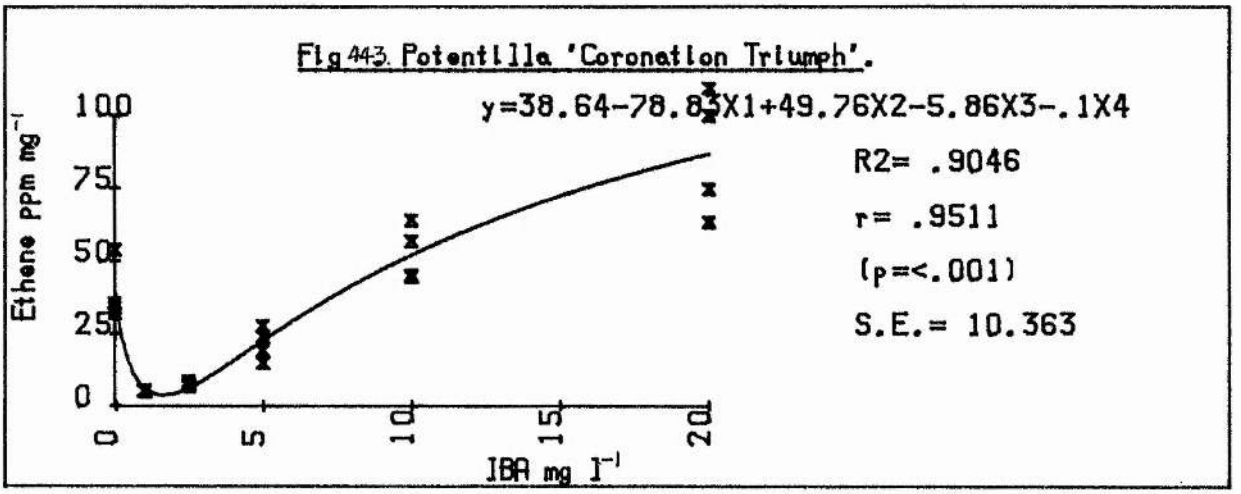
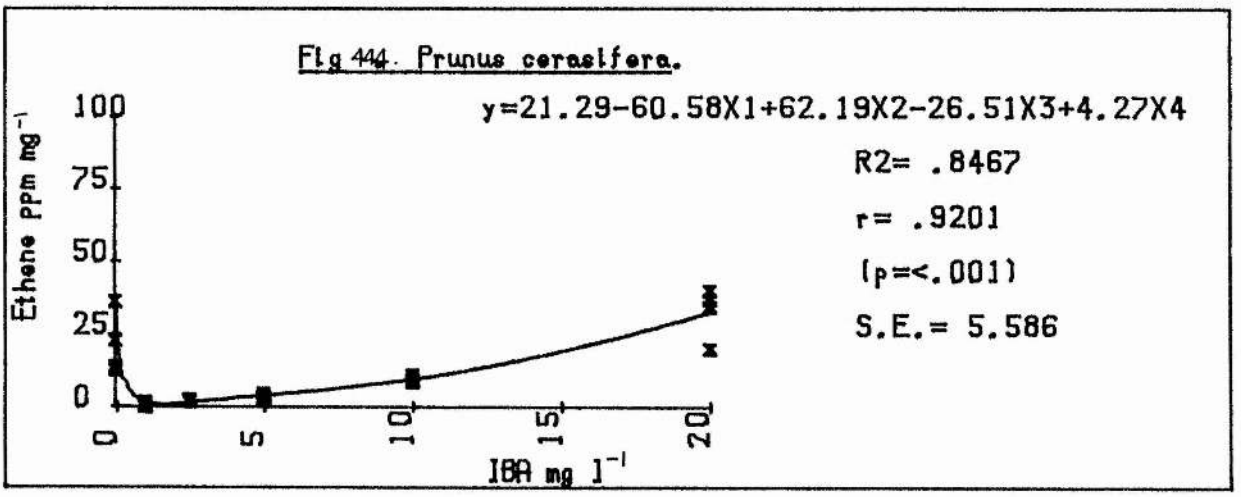


Fig 444. Prunus cerasifera.



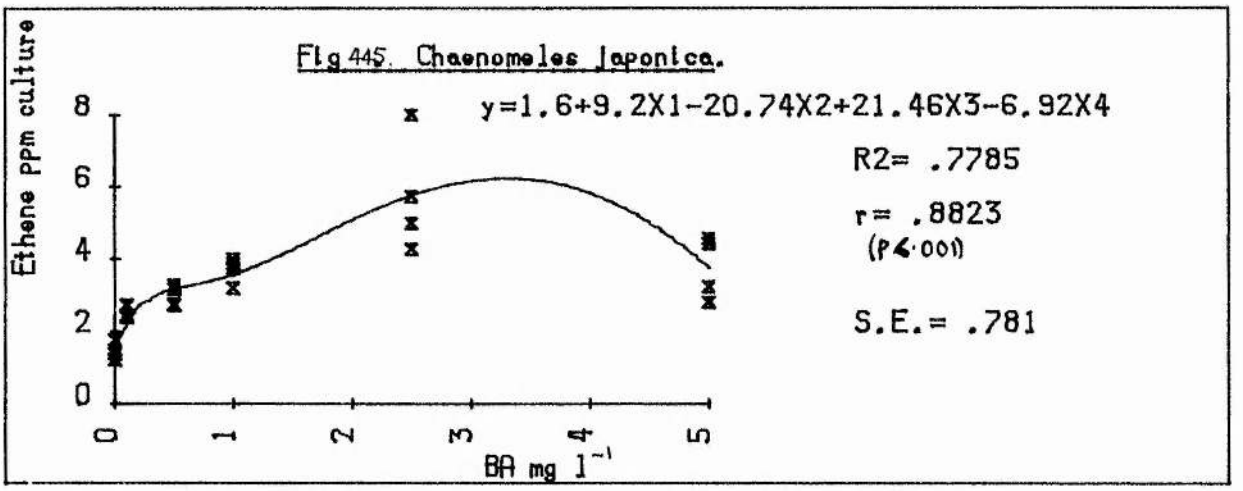


Fig 445. Chaenomeles japonica.
y=1.6+9.2X1-20.74X2+21.46X3-6.92X4
R2= .7785
r= .8823
(P<.001)
S.E.= .781
Ethene ppm culture
BA mg l⁻¹

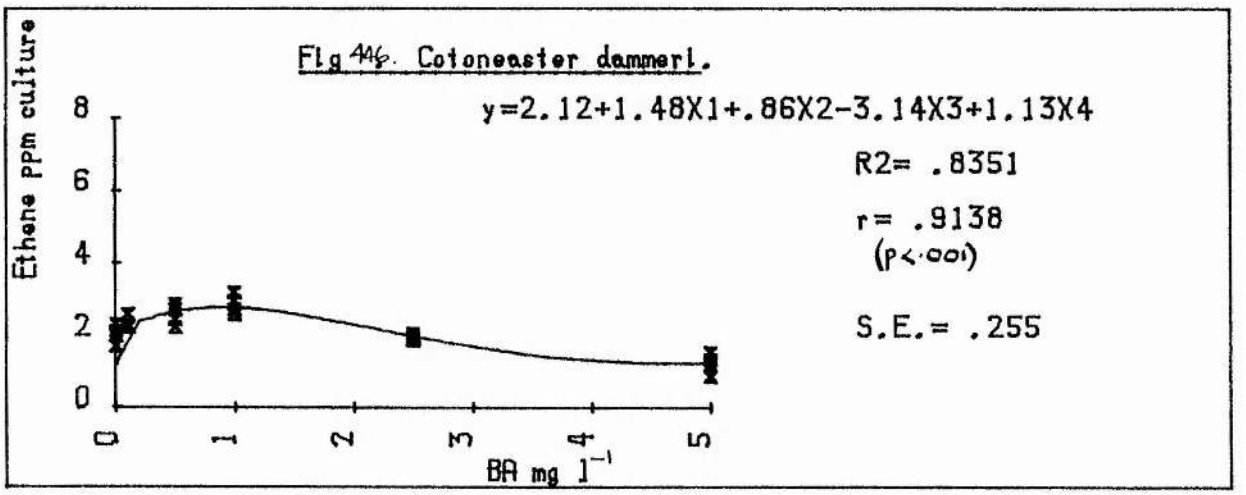


Fig 446. Cotoneaster dammeri.
y=2.12+1.48X1+.86X2-3.14X3+1.13X4
R2= .8351
r= .9138
(P<.001)
S.E.= .255
Ethene ppm culture
BA mg l⁻¹

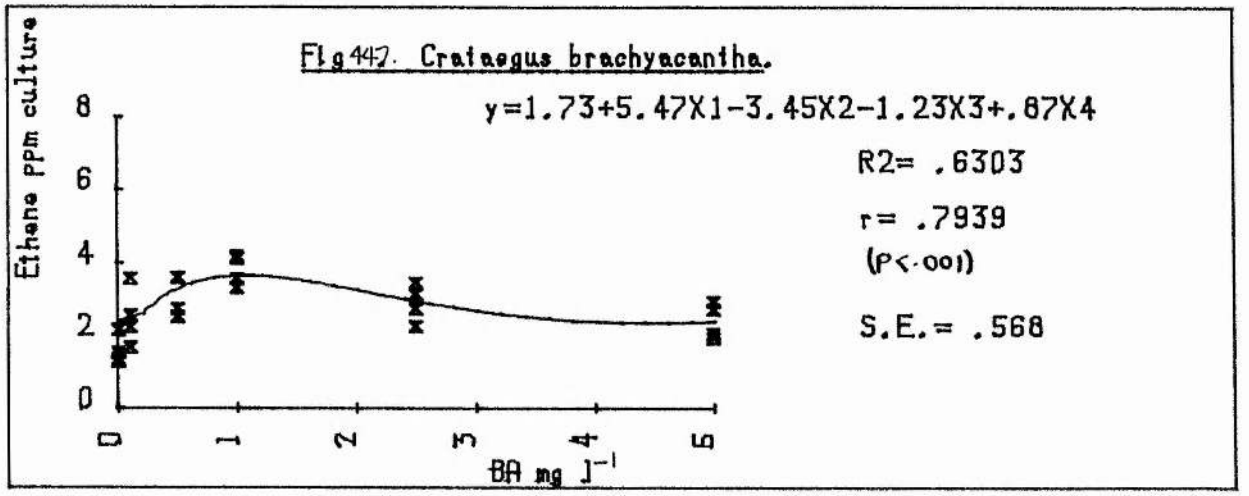


Fig 447. Crataegus brachyacantha.
y=1.73+5.47X1-3.45X2-1.23X3+.87X4
R2= .6303
r= .7939
(P<.001)
S.E.= .568
Ethene ppm culture
BA mg l⁻¹

Fig448 Potentilla 'Coronation Triumph'.

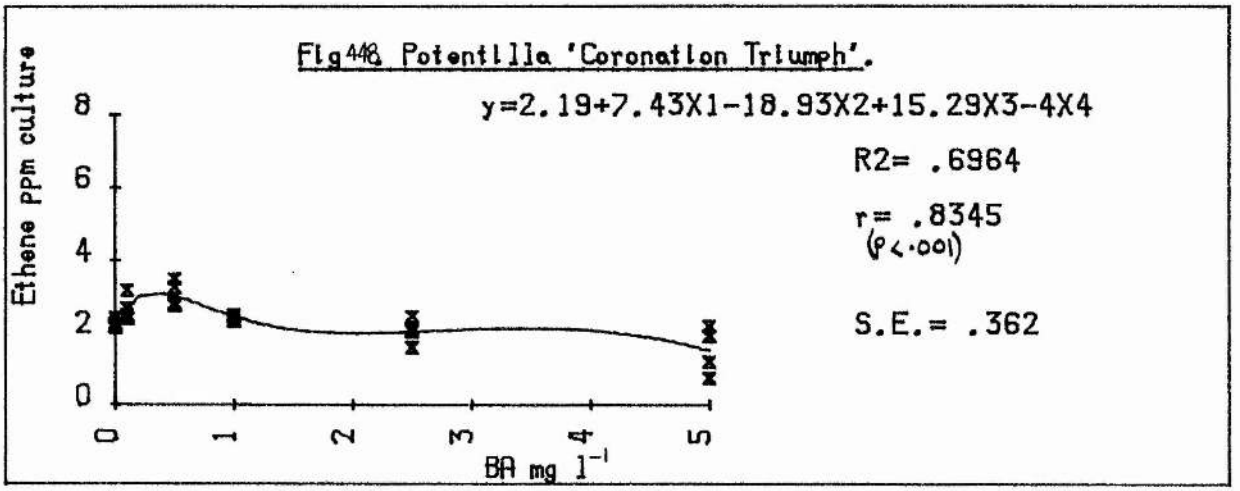


Fig449 Prunus cerasifera.

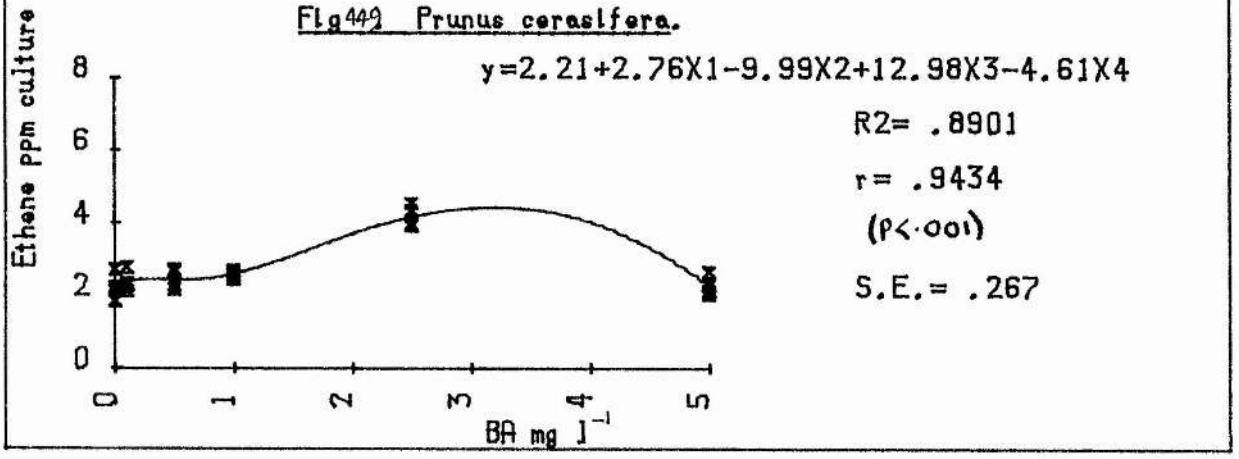
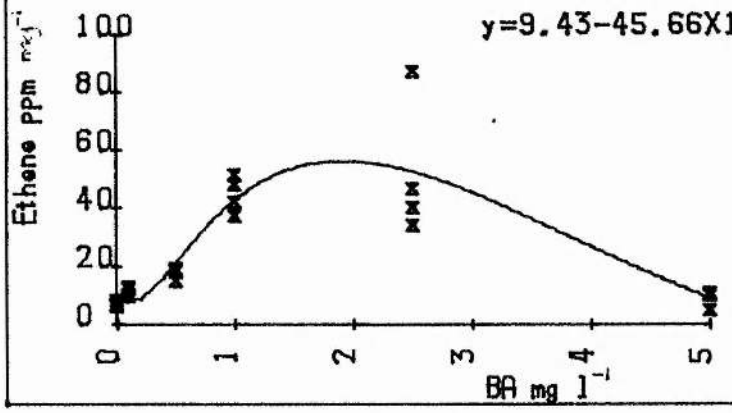


Fig 450 Chaenomeles japonica.



y=9.43-45.66X1+268.67X2-228.01X3+51.45X4

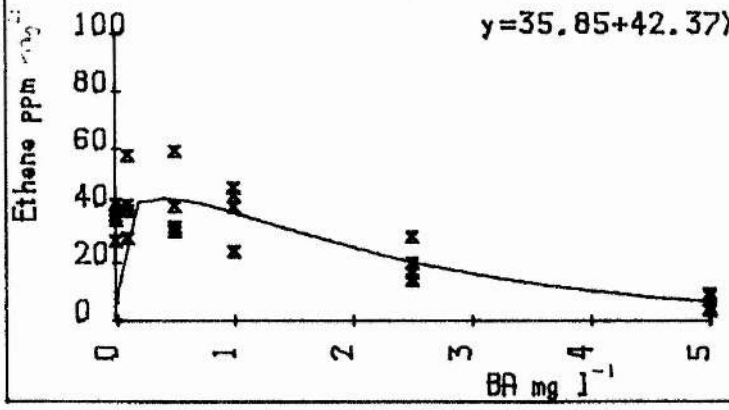
R2= .7879

r= .8876

(p=<.001)

S.E.= 10.324

Fig 451 Cotoneaster dammeri.



y=35.85+42.37X1-80.31X2+35.07X3-4.79X4

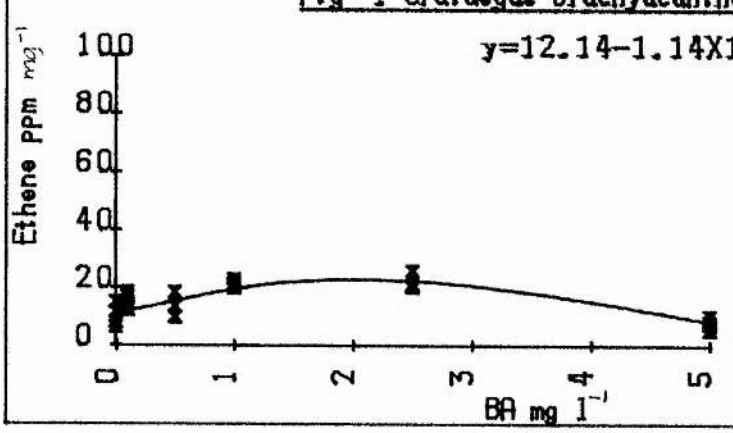
R2= .7202

r= .8486

(p=<.001)

S.E.= 8.9

Fig 452 Crataegus brachyacantha.



y=12.14-1.14X1+32.24X2-23.35X3+2.78X4

R2= .6959

r= .8342

(p=<.001)

S.E.= 3.585

Fig453 Potentilla 'Coronation Triumph'.

y=40.99-165.62X1+370.54X2-290.1X3+72.1X4

R2= .6875

r= .8291

(p=<.001)

S.E.= 7.577

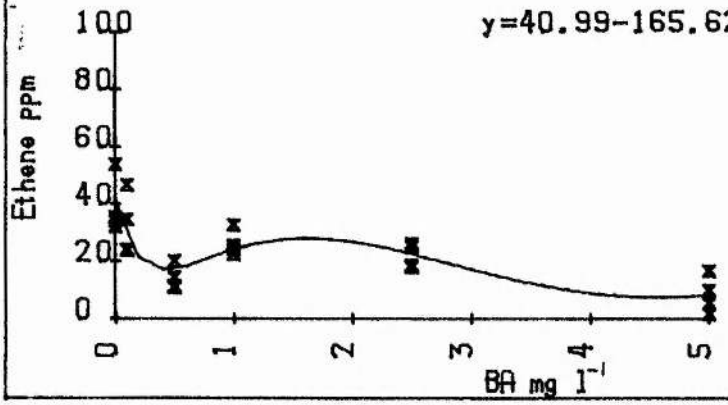


Fig454 Prunus cerasifera.

y=18.24-87.95X1+269.03X2-243.21X3+66.19X4

R2= .6672

r= .8168

(p=<.001)

S.E.= 3.424

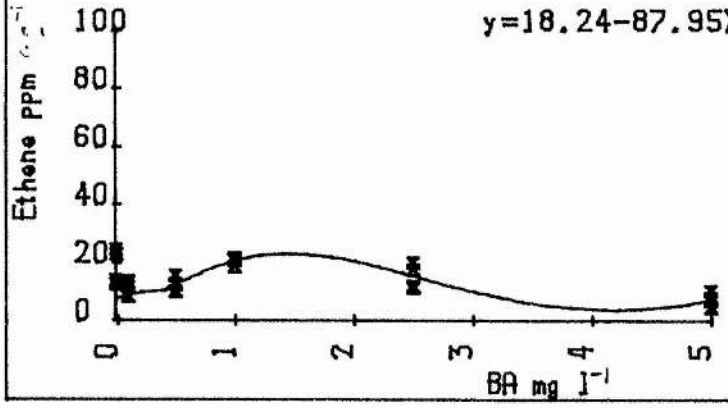
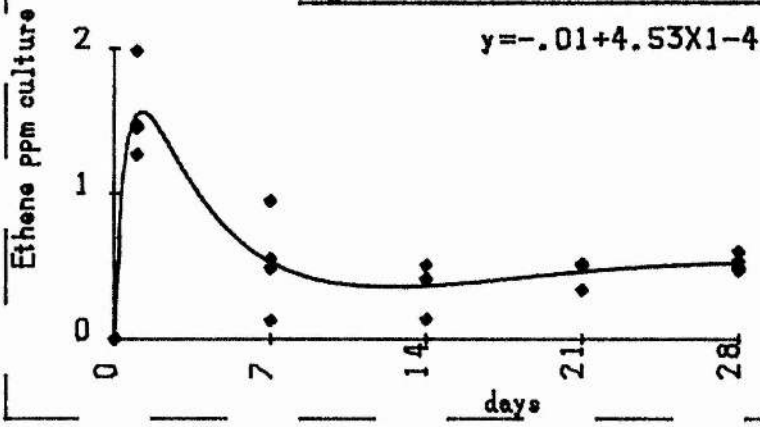


Fig 455. Ethene in control cultures of Prunus cerasifera.



y=-.01+4.53X1-4.09X2+1.1X3-.04X4-.02X5

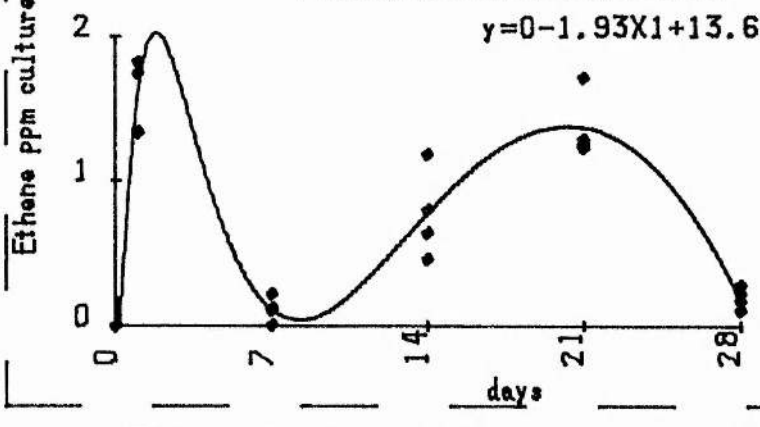
R2= .8786

r= .9373

(p=<.001)

S.E.= .201

Fig 456 Prunus cerasifera BA.



y=0-1.93X1+13.63X2-14.42X3+5.39X4-.68X5

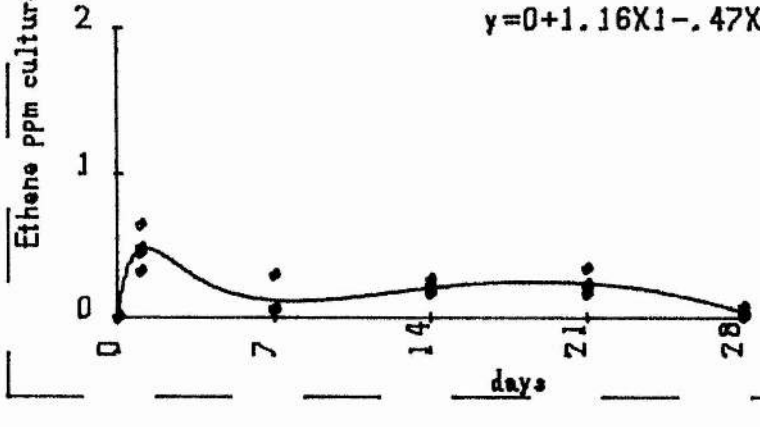
R2= .9296

r= .9641

(p=<.001)

S.E.= .195

Fig 457 Prunus cerasifera IBA.



y=0+1.16X1-.47X2-.53X3+.34X4-.06X5

R2= .8299

r= .911

(p=<.001)

S.E.= .083

Fig458 Ethene in cultures of Prunus cerasifera.

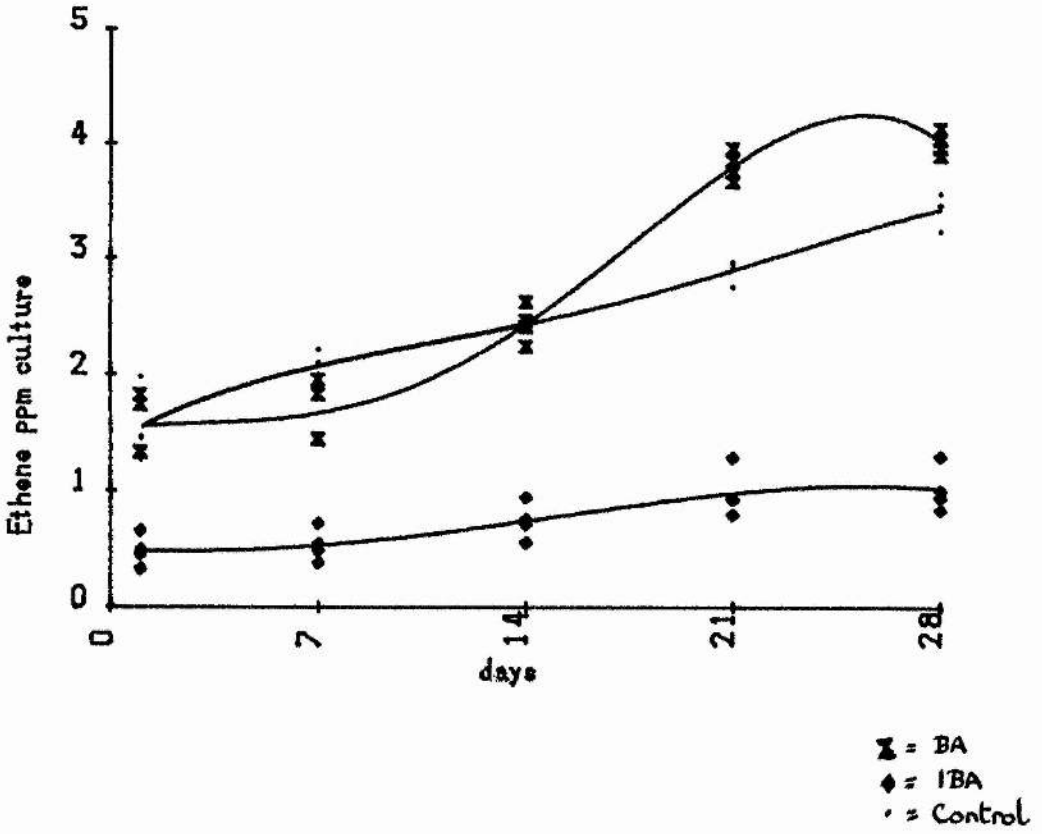


Table 227.

Maximal ethene level (ppm per culture) and optimal BA concentrations for ethene synthesis

	ethene max. (ppm culture)	opt.BA ($mg\ l^{-1}$)
Chaenomeles japonica	6.23	3.30
Cotoneaster dammeri	2.79	0.90
Crataegus brachyacantha	3.66	1.00
Potentilla 'Coronation Triumph'	3.10	0.30
Prunus cerasifera	4.22	3.20

Table 228.

Maximal ethene level (ppm per mg) and optimal BA concentrations for ethene synthesis

	ethene max. ($ppm\ mg^{-1}\ 10^2$)	opt.BA ($mg\ l^{-1}$)
Chaenomeles japonica	56.33	1.90
Cotoneaster dammeri	42.29	0.40
Crataegus brachyacantha	22.91	2.00
Potentilla 'Coronation Triumph'	28.33	0.10
Prunus cerasifera	23.15	1.40

Table 229.

Maximal ethene level (ppm per culture) and optimal IBA concentrations for ethene synthesis

	ethene max. (ppm culture)	opt.IBA (mg l^{-1})
Chaenomeles japonica	5.38	20.00
Cotoneaster dammeri	2.84	20.00
Crataegus brachyacantha	5.82	20.00
Potentilla 'Coronation Triumph'	2.65	20.00
Prunus cerasifera	5.30	20.00

Minimal ethene level (ppm per culture) and least effective IBA concentrations for ethene synthesis

	ethene min. (ppm culture)	IBA low (mg l^{-1})
Chaenomeles japonica	0.442	0.7
Cotoneaster dammeri	0.204	1.2
Crataegus brachyacantha	0.297	1.2
Potentilla 'Coronation Triumph'	0.357	1.7
Prunus cerasifera	0.270	1.0

Table 230.

Maximal ethene level (ppm per mg) and optimal IBA concentrations for ethene synthesis

	ethene max. (ppm mg^{-1} 10^2)	opt.IBA (mg l^{-1})
Chaenomeles japonica	37.17	20.0
Cotoneaster dammeri	61.31	20.0
Crataegus brachyacantha	50.99	20.0
Potentilla 'Coronation Triumph'	87.07	20.0
Prunus cerasifera	39.97	20.0

Minimal ethene level (ppm per mg) and least effective IBA concentrations for ethene synthesis

	ethene min. (ppm mg^{-1} 10^2)	IBA low (mg l^{-1})
Chaenomeles japonica	0.049	0.3
Cotoneaster dammeri	3.03	1.3
Crataegus brachyacantha	2.49	1.8
Potentilla 'Coronation Triumph'	3.58	1.6
Prunus cerasifera	0.647	1.6

Table 231.

Comparison of BA and IBA concentration in ethene synthesis (ppm culture).

conc.(mg l^{-1})

	BA max.	IBA max.	p of diff.
0.5	2.832	1.311	<.001
1.0	3.062	2.412	<.001
2.5	3.358	4.391	<.001

Table 232.

Comparison of BA and IBA concentration in ethene synthesis (ppm mg^{-1}).

conc.(mg l^{-1})

	BA max.	IBA max.	p of diff.
0.5	22.378	11.683	<.001
1.0	26.202	26.049	N.S.
2.5	30.165	53.839	<.001

Table 233.

Analysis of variance for data given in Figures 445 to 449.

SOURCE	S.S.	D.F.	M.S.	F	p
conc.	28.998	5	5.799	24.431	<.001
species	20.297	4	5.074	21.375	<.001
Interaction	46.762	20	2.338	9.849	<.001
Error	21.365	90	0.2374		
Total	117.421	119			

Table 235.

Analysis of variance for data given in Figures 450 to 454.

SOURCE	S.S.	D.F.	M.S.	F	p
conc.	5724.037	5	1144.807	22.008	<.001
species	4237.387	4	1059.347	20.365	<.001
Interaction	9490.117	20	474.506	9.122	<.001
Error	4681.523	90	52.0169		
Total	24133.06	119			

Table 234.

Mean ethene synthesis (combined data for two species) (ppm culture) after BA treatment.

BA conc.	ethene (ppm / culture)
0	1.908d
0.1	2.505c
0.5	2.832b
1.0	3.062b
2.5a	3.358a
5.0	2.233c

Means followed by different letters are significantly different ($p<.05$).

Mean ethene synthesis (combined data for BA concentrations (ppm/culture) after BA treatment.

species	ethene (ppm / culture)
Chaenomeles	3.364a
Cotoneaster	2.178c
Crataegus	2.709b
Potentilla	2.321c
Prunus	2.676b

Means followed by different letters are significantly different ($p<.05$).

Table 236.

Mean ethene synthesis (combined data for two species) (ppm mg^{-1}) after BA treatment.

BA conc.	ethene (ppm / mg)
0	21.878bc
0.1	22.378bc
0.5	19.658c
1.0	30.166a
2.5	26.202ab
5.0	7.906d

Means followed by different letters are significantly different ($p<.05$).

Mean ethene synthesis (combined data for BA concentrations (ppm mg^{-1}) after BA treatment.

species	ethene (ppm / mg)
Chaenomeles	23.702b
Cotoneaster	30.138a
Crataegus	14.991c
Potentilla	23.591b
Prunus	14.398c

Means followed by different letters are significantly different ($p<.05$).

Table 237.

Analysis of variance for data given in Figures 435 to 439.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	209.241	5	41.848	221.105	<.001
species	24.454	4	6.114	32.301	<.001
Interaction	43.486	20	2.174	11.488	<.001
Error	17.034	90	0.1893		
Total	294.215	119			

Table 239.

Analysis of variance for data given in Figures 440 to 444.

SOURCE	S.S.	D.F.	M.S.	F	p
IBA conc.	34858.872	5	6971.774	102.755	<.001
species	8124.261	4	2031.065	29.935	<.001
Interaction	7977.179	20	398.859	5.879	<.001
Error	6106.337	90	67.8481		
Total	57066.651	119			

Table 238.

Mean ethene synthesis (combined data for two species) (ppm/culture) after IBA treatment.

IBA conc.	ethene (ppm / culture)
0	1.908c
1.0	0.317f
2.5	0.828e
5.0	1.311d
10.0	2.412b
20.0	4.392a

Means followed by different letters are significantly different ($p<.05$).

Mean ethene synthesis (combined data for IBA concentrations (ppm/culture) after IBA treatment.

species	ethene (ppm / culture)
Chaenomeles	2.625a
Cotoneaster	1.385c
Crataegus	1.953b
Potentilla	1.419c
Prunus	1.923b

Means followed by different letters are significantly different ($p<.05$).

Table 240.

Mean ethene synthesis (combined data for two species) (ppm mg^{-1}) after IBA treatment.

IBA conc.	ethene (ppm / mg)
0	22.452b
1.0	2.770d
2.5	6.189d
5.0	11.683c
10.0	26.049b
20.0	53.838a

Means followed by different letters are significantly different ($p<.05$).

Mean ethene synthesis (combined data for IBA concentrations (ppm mg^{-1}) after IBA treatment.

species	ethene (ppm / mg)
Chaenomeles	15.079cd
Cotoneaster	22.283b
Crataegus	17.873b
Potentilla	35.447a
Prunus	11.802d

Means followed by different letters are significantly different ($p<.05$).

Table 241.

Analysis of variance for data given in Figures 455 to 457.

SOURCE	S.S.	D.F.	M.S.	F	p
days	23.629	4	5.907	178.201	<.001
treatment	45.261	2	22.630	682.662	<.001
Interaction	7.158	8	0.895	26.991	<.001
Error	1.492	45	0.03315		
Total	77.540	45			

Table 242.

Mean ethene level (combined data for three treatments) (ppm per culture).

days	ethene (ppm / culture)
1	1.192e
7	1.421d
14	1.868c
21	2.559b
28	2.809a

Means followed by different letters are significantly different ($p<.05$).

Mean ethene level (combined data for days) (ppm per culture).

treatment	ethene (ppm / culture)
BA	2.689a
IBA	0.748c
control	2.472b

Means followed by different letters are significantly different ($p<.05$).

Table 243.

Analysis of variance for data given in Figure 458.

SOURCE	S.S.	D.F.	M.S.	F	p
treatment	10.644	5	2.129	74.396	<.001
time	3.182	2	1.591	55.606	<.001
Interaction	4.321	10	0.432	15.101	<.001
Error	1.545	54	0.02861		
Total	19.694	71			

Table 244.

Mean change in ethene level (combined data for three treatments) (ppm per culture).

days	ethene (ppm / culture)
0	0e
1	1.192a
7	0.2516d
14	0.4476c
21	0.6908b
28	0.250d

Means followed by different letters are significantly different ($p<.05$).

Mean change in ethene level (combined data for days) (ppm per culture).

treatment	ethene (ppm / culture)
BA	0.6658a
IBA	0.1798b
control	0.5702a

Means followed by different letters are significantly different ($p<.05$).

8.3 DISCUSSION

Ethephon and morphogenesis

Ethephon has been demonstrated to promote root formation in blueberry (Kender et al., 1969), in Cotoneaster racemiflora (Swanson, 1974) and in Prunus tomentosa (Swanson, 1974). It was not effective in promoting rooting in carnation or Poinsettia (Shanks, 1969), or Chrysanthemum (Carpenter and Carlson, 1972). Geneve and Hauser (1983) and Mullins (1972) showed that rooting was not promoted by ethephon and could be inhibited at high concentrations in mung bean hypocotyl cuttings whereas previous papers (Krishnamoorthy, 1970; 1972; Roy et al., 1972) claimed that ethephon promoted root formation in such cuttings. These reports show conflicting results but may be explained by 1) the use of etiolated or non-etiolated cuutings, 2) different experimental conditions, for example, different relative humidity, and 3) varietal difference in response.

In both species tested in the current work, ethephon promoted root formation. However, non-autoclaved ethephon failed to promote rooting at concentrations above 5 mg l^{-1} .

Ethephon promoted rooting to a lesser extent than IBA in Spiraea suggesting that ethene is not acting as a mediator for all IBA activity in root formation in this species. However, Prunus exhibited significantly greater rooting in ethephon than in IBA treatments. Incubation in this experiment was in light and it was previously demonstrated (Chapter 4) that IBA promoted rooting in Prunus only when explants are incubated in darkness. This experiment shows that darkness is not essential for rooting in Prunus. Therefore, the dark requirement is likely to be related to IBA action in root promotion.

Shoot formation has also been demonstrated to be promoted by ethephon in a few species (e.g. Sedum - Boe et al., 1972; Geranium - Carpenter and Carlson, 1972a). Shoot formation was promoted in both species in the current experiment.

Shoot number was significantly lower at the optimal ethephon concentration than in the optimal BA treatment. Therefore, ethene is unlikely to be acting as a mediating compound in this instance. However, ethene probably accounts for some of the BA-promoted shoots.

Shoots formed were axillary in origin, whereas BA

promoted the formation of axillary and adventitious shoots. Therefore, ethene may be acting as an intermediate in formation of axillary but not adventitious shoots.

Ethephon promoted simultaneous shoot and root initiation (Plate 20) whereas cytokinin and auxin, when applied individually, promoted differentiation to a greater extent but in only one direction. Ethephon, therefore, appears to mimic the response to cytokinin and auxin simultaneously. This response could indicate that applied ethene is stimulating endogenous auxin and cytokinin activity.

Ethene has been shown both to reduce and to increase endogenous levels of auxin in pea seedlings (Burg et al., 1971), the effect of ethene varying with the part of the plant; polar transport of auxin can also be reduced (Goldsmith, 1977); and IAA oxidase activity can be increased by ethene (Hall and Morgan, 1964). In addition, ethene has been shown to promote polyphenoloxidase and peroxidase / IAA oxidase activity (Adams and Galston, 1974). Thus, ethene application could affect auxin concentration at the site of action for morphogenesis.

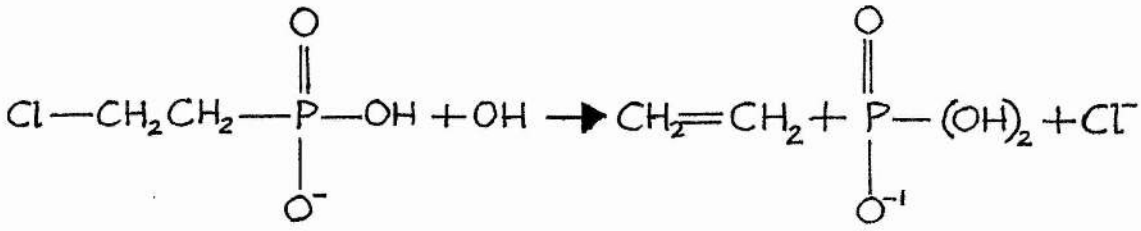
Interaction with endogenous cytokinin has also

been reported. Banko and Boe (1975) found that ethephon at a concentration of 10 ppm reduced cytokinin activity in Coleus. If cytokinin levels were reduced in Prunus and Spiraea after ethephon application, then ethephon cannot be promoting shoot formation via cytokinin but must be acting in some other way. No callus was formed in ethephon or ethene treatments, but callus did form when auxin or cytokinin was present in the medium (see Chapter 5) - this is additional evidence that ethene / ethephon action in differentiation is unlikely to be mediated by auxin or cytokinin. Linkins et al. (1973) suggested that ethene reduces the mechanical resistance of the cortex to the emergence of root initials by enhancing cellulase activity. This could also be important in promoting the emergence of shoot initials.

The results reported here contradict results from other <u>in vitro</u> studies where organized growth and differentiation was found to be inhibited by ethephon (Wochok and Wetherell, 1971; Hsu and Stewart, 1976). Hsu and Stewart (1976) showed that in addition to inhibiting differentiation of cotton ovules, ethephon promoted callus formation. Callus formation in the presence of ethephon has also been reported in citrus

bud cultures (Goren et al., 1979). My results show the reverse - no callus was formed and differentiation was enhanced.

It is possible that the observed effects on morphogenesis are not due to ethene but rather can be attributed to the phosphate residue product of ethephon breakdown.



Raising the phosphate concentration of the medium has been shown to increase shoot formation in the presence of a cytokinin and decrease root formation in the presence of an auxin (Reinert et al., 1977). However, in the present study both shoot and root formation were enhanced and no exogenous cytokinin or auxin was applied. In defence of the observed results being principally caused by ethene, attention can also

be drawn to the experiment conducted with ethene gas in which shoot and root formation were enhanced. The observed differences in effect between autoclaved and non-autoclaved ethephon may be due to premature release of ethene from the autoclaved samples. Ethene release from ethephon principally occurs outside the plant tissue (Mudge and Swanson, 1978). Ethene concentrations would therefore be lower in autoclaved cultures. The fact that only low concentrations of unautoclaved ethephon promoted morphogenesis would seem to support the hypothesis that low ethene concentrations do promote morphogenesis in the test species.

Ethene synthesis

Both auxin and cytokinin enhanced ethene synthesis. However, the pattern of production in relation to auxin or cytokinin concentration was different.

An increase in ethene synthesis with increase in IBA concentration was recorded. However, when IBA concentration was low, ethene levels were lower than in control cultures (no added auxin). This demonstrates, therefore, that auxin inhibits ethene synthesis at low

concentrations but increases ethene synthesis at high concentrations.

It has been known for some time that auxin regulates ethene production (Zimmerman and Wilcoxon, 1935). More recently, other work has shown similar results. For example, Morgan and Hall (1962, 1964) showed that plants sprayed with 2,4-D or IAA produced an increased level of ethene; and Gavinlertvatana et al. (1982) demonstrated greater stimulation of ethene synthesis at 10 mg l^{-1} than at 1 mg l^{-1} in dahlia callus. The rate of formation of ACC (1-aminocyclopropane-1-carboxylic acid) from SAM (S-adenosylmethionine) is affected by auxin supply (Adams and Yang, 1979). ACC is the immediate precursor of ethene. Thus, the higher the auxin concentration, the more rapid would be the expected rate of ethene synthesis.

For my results, however, this supposition does not hold. One would expect the auxin concentration in shoot explants which had no exogenous auxin supplied to them to be lower than in those which were supplied with auxin. Therefore, one would expect more ethene synthesis when exogenous auxin is applied. This was not shown to occur.

It can be assumed, therefore, that the exogenous auxin is being channelled for some purpose other than ethene synthesis. This other purpose could be growth or differentiation. Shoot growth was stimulated to the greatest extent at low auxin concentrations and rhizogenesis occurred mainly at intermediate auxin concentrations. Therefore, auxin activity at these levels may be diverted towards growth and differentiation.

Alternatively, ethene synthesis might not be the direct result of the presence of auxin. Change in ethene levels have been shown to be associated with tissue transition (Gavinlertvatana et al., 1982) and in particular with the rate of cell division (Mackenzie and Street, 1970; LaRue and Gamborg, 1971). Ethene synthesis could therefore be a result of auxin-stimulated growth and differentiation, rather than as a direct result of auxin concentration.

Examples of plants in which increases in ethene synthesis following cytokinin application have been reported are bean (Abeles et al., 1967), blueberry (Forsyth and Hall, 1968) and mung bean (Imaseki et al., 1982). Cytokinins have been shown to control ACC formation from SAM (Adams and Yang, 1979) by

controlling ACC synthase activity (Imaseki et al., 1982). BA is also thought to increase the availability of ACC to the synthetic site of ethene (Imaseki et al., 1982).

When supplied wth cytokinin, ethene levels were greatest at the BA concentrations which promoted most shoot formation. This evidence supports the hypothesis that ethene is produced as a result of cell division rather than as a direct consequence of cytokinin concentration.

Ethene synthesis was greatest in the first day of culture on media containing auxin, cytokinin or no growth regulator. This period of high ethene synthesis occurs immediately after excision of the explants and is probably a result of wounding (Osborne, 1978).

Auxin and cytokinin enhanced ethene production but did not alter the pattern of its synthesis with time. This was also reported in carrot root tissue cultures (Bender and Neumann, 1978). They found peak production to be during the first five hours and on the sixth day when IAA or IAA + kinetin was given. Gavinlertvatana et al. (1982) reported an overall increase in ethene levels with time, and an increase in the third week concurrent with visible callus formation. In my

experiments, ethene synthesis peaked from day 24 to 28 depending on treatment and then declined. This suggests a relationship with growth rate.

IX. DISCUSSION

IX. DISCUSSION

The role of excision and cell division in differentiation

Axillary shoots were formed on intact plants and excised shoots. Axillary shoots derive from buds already present and therefore dedifferentiation is not necessary for their formation. However, neither callus nor adventitious organs formed on intact plants. This suggests that excision or wounding may be necessary for dedifferentiation. De novo bud formation without excision was reported in intact attached leaves of Begonia after 2iP treatment (Chlyah-Arnasson and Tran Thanh Van, 1968), but this appears to be an isolated case.

Wounding of plant tissues leads to changes in metabolism. Respiration rate and ethene synthesis rapidly increase (Tran Thanh Van, 1981); phenolic compounds, for example, chlorogenic acid (Kuc, 1972), are metabolized (Rhodes and Wooltorton, 1978); and an increase in membrane permeability, synthesis of DNA and proteins, and increase in peroxidase activity have also been reported (Tran Thanh Van, 1981). These changes

could affect differentiation by changing endogenous growth regulator status, for example, phenolic compounds may (a) act synergistically with auxins by inhibiting IAA oxidase activity or promoting IAA synthesis (by promoting tryptophan synthesis from anthranilic acid) (polyphenols), or (b) promote IAA oxidase activity and thus IAA breakdown and inhibit IAA synthesis via the tryptophan pathway (monophenols) (Kefeli and Kutacek, 1977).

Mechanical wounding causes increased cell division activity (Galston and Davies, 1970). As differentiation is usually associated with meristematic areas, it has been thought that cell division is closely associated with differentiation (Jeffs and Northcote, 1966, 1967). Fosket (1968) showed that cytodifferentiation only occurred immediately after cell division, and tracheids originate from actively dividing cells of callus cultures (Torrey 1975). Ho wever, Kohlenbach and Schmidt (1975) showed that cell division is not an absolute requirement for cytodifferentiation - mesophyll cells of Zinnia could be transformed into tracheids without preceding cell division or mitotic activity; and Foard and Haber (1961) showed that differentiation in irradiated wheat

seedlings could occur without cell division.

Shoots, roots, and callus did not form in response to excision alone - addition of auxin or cytokinin was necessary. This has also been shown for tobacco callus (Skoog and Miller, 1957). Although not necessarily an absolute requirement for differentiation, meristematic activity has been shown to precede initiation of roots and shoots (Dodds and Roberts, 1982). Street (1977) proposed that the meristematic region formed by cell division, could act as a sink which may withdraw metabolites from surrounding cells (thus localizing the meristematic zone). Excision may therefore precondition plant cells for differentiation by promoting cell division.

The role of light in differentiation

Light promoted shoot formation and cytokinin-stimulated callus formation, whereas darkness promoted auxin-stimulated root and callus formation. A correlation of light with growth regulator activity therefore is apparent.

Light induces proton extrusion from plant cells (Van Volkenburgh and Cleland, 1980; Gepstein, 1982) - this effect is a direct effect of light rather than an

indirect effect via auxin. This may explain the promotionary effect of light on growth, but does not explain the observed effects on differentiation as growth occurred both in light and in darkness.

Exogenous auxin was essential for root and callus formation in darkness. Its function could be due, at least in part, to its promotionary effect on proton secretion (reduced by darkness). However, if the only effect of auxin on differentiation was in promotion of hydrogen ion secretion, then, it would be expected that root formation would occur in light in the absence of auxin. This did not occur. Hydrogen ion secretion in light might promote shoot formation, but auxin did not promote shoot formation in darkness. These results suggest that, while hydrogen ion secretion may be necessary for differentiation (differentiation involves growth), hydrogen ion extrusion does not determine the direction of differentiation.

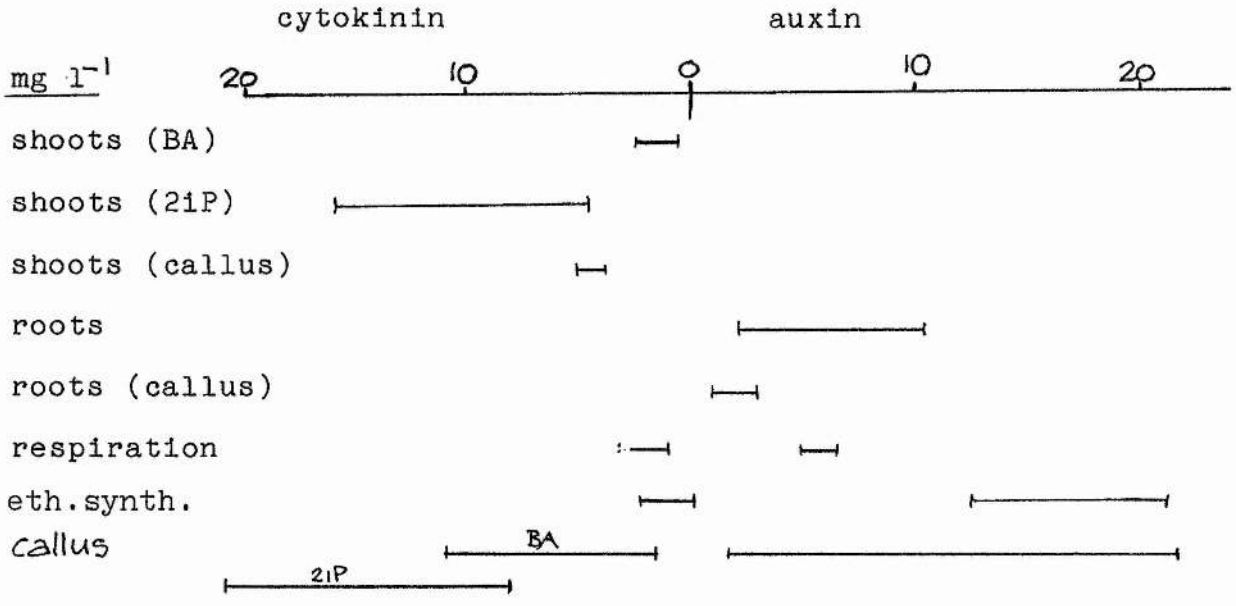
Red light promotes cytokinin synthesis (Letham, 1978), and this could explain the greater callus formation in darkness than in light (both auxin and cytokinin being present, thus allowing cell division and cell growth to occur). Light influences cell division (Roberts, 1976), but enhanced cell division is

unlikely to determine the direction of differentiation (see above). Other known effects of light include decrease of rate of transport (Thimann, 1977) and alteration of microtubule orientation with a resultant effect on cell wall growth (Miller and Stephani, 1971). These could affect differentiation indirectly.

Although wavelength of light has been shown to be important in differentiation (Seibert et al., 1975), phytochrome action has not been shown to have an effect (Roberts, 1976).

Auxin and cytokinin concentration

Auxin and cytokinin concentrations most active in promotion of shoot, root and callus formation, and respiration and ethene synthesis, are given below.



In general, callus formation occurred at higher auxin and cytokinin concentrations than did root and shoot initiation. Shoot and root formation from callus required a higher exogenous growth regulator concentration than shoot and root formation from shoot explants. However, organogenesis from callus took a longer time to occur, and therefore, the high external concentration may have been decreased by the time organogenesis occurred. Uptake of BA can be very rapid, and in Jerusalem artichoke tuber slices on Murashige and Skoog (1962) medium, uptake after 30 to 45 minutes was occurring against a concentration gradient. Degradation begins after 30 minutes of incubation (Minocha and Nissen, 1982). Aloni (1980) found that different concentrations of IAA applied to callus, promoted a different pattern of cytodifferentiation - a low concentration promoted sieve elements but no tracheary elements, and a high concentration of IAA led to phloem and xylem formation. This could explain the observed failure of callus to differentiate at low auxin concentrations.

The amounts of exogenous cytokinin and auxin necessary for organogenesis have been thought to depend on the availability of their endogenous counterparts

(Hussey, 1978b), and correlations between auxin content of the tissue and growth have been made (Leopold, 1955; Wightman, 1977). Endogenous factors affected differentiation substantially. The shoot apex and lateral buds contained factors which promoted root and callus formation but inhibited shoot formation. However, shoot, root and callus formation were all greater in explants derived from the top of the plant, and all followed a similar pattern with season. These effects could not be overridden by adjustment of exogenous auxin or cytokinin, and therefore, these results suggest that it is not simply growth regulator status which affects differentiation.

The curves for concentration and response show a very wide concentration range of auxin or cytokinin to be active in axillary shoot formation, root formation, callus formation, ethene synthesis and respiration rate. These curves are similar to dose-response curves reported by others in that they do not obey Michaelis-Menten kinetics. However, two responses occurred within a very narrow concentration range - adventitious shoot formation, and chloroplast formation in callus. This suggests that growth regulator concentration acts as a trigger in these narrow

concentration range responses, but not in the other responses.

Dose-response curves may parallel binding of the growth regulator molecules. Wide dose responses may result from a step-wise lowering in affinities (due to change in rate of growth regulator uptake) with which the growth regulators bind to the carrier or the receptor protein (Minocha and Nissen, 1982), and may also reflect changes in properties of the receptors (Rubery, 1981).

A variety of responses, both in morphogenesis and metabolism, to auxin and cytokinin was observed. This may reflect action at separate sites or may be the result of one or more regulatory responses. Growth regulators are believed to affect a single target, all responses being derived from this initial interaction. If growth regulators combine with protein receptor sites associated with membranes (Letham et al., 1978), then the resultant molecule, if released from the membrane, could act in different parts of the cell including the nucleus (Hardin et al., 1972). Thus, the same molecule might act both on the nucleus to control gene expression and at other sites to control metabolism.

Some of the observed responses appeared to be linked. Cytokinin-enhanced axillary shoot formation, ethene synthesis and oxygen uptake (respiration) varied similarly with concentration. From their work on suspension cultures of Rosa, however, LaRue and Gamborg (1971) suggested that ethene synthesis and respiration are independent processes, and that auxin could stimulate ethene synthesis without affecting respiration. This did not appear to be so in the current experiments. Other evidence suggests that ethene synthesis and respiration may be linked. Chalmers and Rowan (1971) provided evidence that regulatory enzymes in glycolysis were affected by ethene, and Solomos and Laties (1976) showed that ethene diverted electron transport from the cytochrome pathway to an alternative cyanide-insensitive electron transport system. This may indicate that cytokinin is controlling all of these responses by acting at one site.

Species differences in morphogenesis

Species differences were apparent in (1) the growth regulators which induced a response (BA promoted shoot formation in Rosaceae, whereas 2iP was more

effective in Ericaceae), (2) the concentrations which were effective to produce the same degree of differentiation, and (3) the degree of differentiation which took place. These differences could not be accounted for by differing quantities of endogenous auxin or cytokinin as a range of concentrations was supplied and a difference was still evident. Armstrong et al (1981) reported both intra- and inter-specific diversity in cytokinin metabolism in Phaseolus. The two plant families studied here differed markedly in their response to cytokinin, but less so to auxin.

In general, species which readily formed shoots, also readily formed roots both in vitro and in greenhouse conditions. However, callus formation increased as shoot and root formation decreased. Species which readily differentiated, did not easily form callus and vice versa.

Genetic character substantially affects differentiation and response to growth regulators and does not seem to be readily changed.

Change in differentiation after repeated subculture

Repeated subculture resulted in reversible morphological changes (phenotypic changes) and

apparently irreversible changes in morphogenic potential. This change in morphogenic potential appears to be related to cytokinin since a difference in the extent of the decline promoted by BA and 2iP was recorded. This change was predictable and progressive. A similar change has been observed to occur in some callus cultures which become cytokinin habituated. This is also a gradual progressive decline, and is thought to be an epigenetic change, i.e. a change involving heritable cellular alterations without permanent change in the genome (Meins, 1983).

Epigenetic change involves selective gene expression and occurs regularly in response to specific inducers, in this case probably cytokinin. Fluxes in growth regulator, mineral or sucrose content of the medium could also affect this type of change. Disappearance of carbohydrate from the medium occurs rapidly - in suspension cultures of Rosa, the total carbohydrate of the medium (2% sucrose) disappeared by day 8 to 12 depending on the density of the inoculum (Nash and Davies, 1972). A similar rate of carbohydrate depletion has also been recorded for Ipomoea (Rose et al., 1972). The rate of BA uptake is very rapid and depends on BA concentration in the

external medium (Minocha and Nissen, 1982), high concentrations promoting more rapid uptake than low concentrations. IAA binding protein levels may also rise and decline during each subculture period (shown for tobacco callus - Oostrom et al., 1980).

As both root and shoot formation declined with subculture, this suggests that linked genes (possibly repressed in this instance) control both root and shoot formation. The situation appears different, however, in callus cultures of the same species, as the capacity for shoot formation appeared to be lost in culture while the capacity for root formation was retained - these results suggest different genes for shoot and root formation. It is possible that some genes control both shoot and root formation and others control only one or the other.

X. SUMMARY

X. SUMMARY

1. Shoot, root and callus formation in a range of Rosaceous and Ericaceous species were recorded after in vitro culture with auxin or cytokinin. Exogenous cytokinin was necessary for shoot formation, exogenous auxin for root formation, while callus formation occurred in the presence of exogenous auxin or cytokinin.

2. A difference in activity in shoot induction between BA and 2iP was recorded - BA induced many short shoots whereas 2iP induced fewer longer shoots. It is proposed that oxidation of 2iP, and BA-stimulated ethene synthesis account for these differences.

3. A difference in activity in callus formation was recorded between the cytokinins BA, 2iP and kinetin, and between the auxins IBA, NAA and 2,4-D. 2,4-D was the most active auxin; the most active cytokinins were BA in Rosaceae and 2iP in Ericaceae.

4. Auxin-induced callus and root formation was promoted by darkness whereas cytokinin-promoted callus

formation was stimulated in light. When auxin and cytokinin were applied simultaneously, callus growth was enhanced. Sequential treatment showed that callus formation was stimulated when cytokinin was supplied before auxin but was inhibited when auxin was given before cytokinin.

5. Four different callus types occurred in response to a) auxin and cytokinin applied, b) auxin and cytokinin concentration , c) light and temperature.

6. Repeated subculture of shoots on medium containing cytokinin resulted in an initial rise, followed by a decline in caulogenesis with propagative generation; and a decline in root formation and an increase in callus formation. This decline was apparently irreversible.

7. A decrease in shoot length and leaf size occurred with repeated subculture. These changes could be partially reversed by a) auxin, b) gibberellin, c) decreased illumination.

8. Oxygen consumption (respiration rate) and ethene

synthesis followed a similar pattern in relation to concentration of growth regulator applied. Cytokinin-enhanced respiration and ethene synthesis may be related to shoot formation, but auxin-promoted respiration and ethene synthesis was not correlated with root initiation.

9. Ethephon and ethene promoted shoot and root formation simultaneously in light, but ethene is unlikely to act as an intermediate in the promotion of all auxin- and cytokinin-induced differentiation.

10. Axillary shoots were formed in all species in response to BA, 2iP, or ethene in intact plants and excised plant parts. Adventitious shoots formed in most Rosaceous species but only in a few Ericaceous species in response to BA, excision and / or in vitro culture.

11. Elimination of potassium from the medium resulted in an increase in root formation but a decrease in shoot and callus formation.

12. A high hydrogen ion concentration (low pH)

promoted root and callus formation, while a higher pH promoted shoot formation.

13. Endogenous factors were important in determining the direction of differentiation. Axillary buds and the shoot apex promoted root and callus formation but inhibited shoot formation.

14. Species differences were evident in morphogenetic response to cytokinin and auxin, in the growth regulator concentrations required for morphogenesis, and in the extent of morphogenesis.

XI. APPENDIX

11.1 Sources of plants

The following botanical gardens, arboreta and nurseries supplied plants or cuttings for use in the experiments detailed in this thesis :-

1) University of St. Andrews Botanic Garden, St. Andrews, Scotland. (St.A)
2) University of Idaho Plant Sciences Farm, Moscow, Idaho, U.S.A. (ID)
3) North Willamette Research Station, Oregon State University, Oregon, U.S.A. (OR)
1) University of Washington Arboretum, Seattle, Washington, U.S.A. (WA)
5) National Arboretum, Washington, D.C., U.S.A. (NA)
6) Garden Square, Lewiston, Idaho, U.S.A. (L)

Ericaceae

Arctostaphylos media	OR
Arctostaphylos uva-ursi	ID
Erica carnea 'Springwood White'	ID
Gaultheria hispidula	WA
Kalmia angustifolia	OR
Rhododendron arboreum	WA

R. chamae-thomsonii	WA
R. 'chikor'	OR
R. 'chinsayii'	OR
R. concinnum	St.A.
R. dauricum	WA
R. fastigiatum	L
R. forrestii	WA
R. keiskei	NA
R. leucaspis	WA
R. lutescens	WA
R. 'P.J.M. Victor'	OR
R. ponticum - collected wild in	Fife
R. racemosum	WA
R. 'Vuyk's rosy red'	L
R. williamsianum	
Vaccinium vitis-idaea 'minus'	WA

Rosaceae

Chaenomeles japonica	ID
Cotoneaster dammeri 'Mooncreeper'	OR
Crateagus brachyacantha	OR
Crataegus X mordenensis 'Toba'	ID
Malus 'Dainty'	ID
Malus 'Golden Hornet'	ID

Potentilla fruticosa 'Coronation Triumph'	ID
Potentilla fruticosa 'Sutter's Gold'	ID
Prunus cerasifera 'Thundercloud'	ID
Prunus tomentosa	ID
Pyracantha coccinea 'Lalandei'	ID
Spiraea X *bumalda* 'Froebelii'	ID

11.2 pH AND AGAR GELATION

The extent of gelation of a medium containing agar is in part determined by the pH of the medium, for example, in highly acid media, gelation may not occur (Murashige, 1973a). This is due to the fact that agar is hydrolyzed below pH 6 when heated (Burnett et al., 1957). In some experiments described in this thesis, it was necessary to vary the pH between 3 and 10. Experiments were therefore conducted to determine the gelling properties of agar over the range of acidity required.

11.21 pH adjustment prior to autoclaving

Method

LS medium was made up containing varying concentrations of Difco Bacto agar. The concentrations used were 5, 6, 7, 8, 9, or 10 g l^{-1}. The pH of the media was adjusted after heating the solution until the agar had dissolved. The pH values tested were 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5,

9.0, 9.5, 10.0. The solutions were then autoclaved, and the number gelled (of three replicates per treatment) counted when the solutions had cooled to room temperature. The agar was considered to have gelled if a stem explant of the type used in the experiments did not sink when laid on the surface of the medium.

Results

The results are given in Table 245. At pH 3 and 3.5, gelation did not occur; above pH 6, all media gelled; between pH 4 and 5.5, extent of gelation varied with pH and agar concentration. Above pH 6, a very hard gel resulted when high concentrations (8 to 10 g l^{-1}) of agar were used.

11.22 pH adjustment after autoclaving

Method

Agar media were made up as above but the pH was not corrected (it was maintained at approximately 7.0).

The solutions were autoclaved, and then allowed to cool to differing temperatures before the pH was adjusted to the required value. The temperatures at which pH adjustment was made were 90, 80, 70, 60, 50, 45, 40, 35, 30 and 25°C. pH was adjusted to 3, 3.5, 4, 4.5, 5, or 5.5. Agar concentration was 7 or 10 g l^{-1}. The number of solutions which gelled (of three replicates for each treatment) was recorded when the solutions had cooled to room temperature.

Results

The results are given in Tables 246 and 247. Gelation was optimal at 35°C. At this temperature, a clear gel was obtained. At higher temperatures, gelation was sporadic, and at lower temperatures, bubbles formed in the gel and gelation was poor because mixing of the solution took place during setting.

N.B. Other brands of agar may have different gelling characteristics, for example, agar from Flow Laboratories gives a harder gel at low concentrations.

Table 245.

Agar gelation in relation to pH

Number gelled (of three replicate solutions)

pH	concentration of agar (g l^{-1})					
	5	6	7	8	9	10
3.0	0	0	0	0	0	0
3.5	0	0	0	0	0	0
4.0	0	0	0	0	1	3
4.5	0	0	1	3	3	3
5.0	0	2	3	3	3	3
5.5	0	3	3	3	3	3
6.0	3	3	3	3	3	3
6.5	3	3	3	3	3	3
7.0	3	3	3	3	3	3
7.5	3	3	3	3	3	3
8.0	3	3	3	3	3	3
8.5	3	3	3	3	3	3
9.0	3	3	3	3	3	3
9.5	3	3	3	3	3	3
10.0	3	3	3	3	3	3

Table 246.

Agar gelation in relation to temperature of pH adjustment

(7 g l^{-1} agar)

Number gelled (of three replicates)

°C	pH					
	3.0	3.5	4.0	4.5	5.0	5.5
25	1	2	2	2	3	3
30	2	2	3	3	3	3
35	3	3	3	3	3	3
40	1	2	3	3	3	3
45	0	0	1	1	3	3
50	0	0	0	1	3	3
60	0	0	0	0	3	3
70	0	0	0	1	3	3
80	0	0	0	2	3	3
90	0	0	0	0	3	3

Table 247.

Agar gelation in relation to temperature of pH adjustment

(10 g l^{-1} agar)

Number gelled (of three replicates)

°C	pH					
	3.0	3.5	4.0	4.5	5.0	5.5
25	1	2	1	3	3	3
30	2	3	3	3	3	3
35	3	3	3	3	3	3
40	2	3	3	3	3	3
45	0	1	3	3	3	3
50	0	0	0	3	3	3
60	0	0	0	2	3	3
70	0	0	0	1	3	3
80	0	0	0	1	3	3
90	0	0	0	1	3	3

11.3 THE EFFECT OF EXPLANT SIZE ON SURVIVAL

Often a different formulation of nutrients and growth regulators must be supplied to very small explants in order that the explant can be provided with the factors necessary for healthy growth. Some of these factors may be present in larger explants but may not be present in sufficient quantity in very small explants. An experiment was conducted to assess the effect of explant size on survival on Anderson (1975) (A1) medium in two Rhododendron species.

Method

Explants of Rhododendron concinnum and Rhododendron ponticum were cut to varying lengths after sterilization and bark removal. The lengths used were 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 mm. The number of explants which survived after one week on an Anderson (1975) (A1) medium was recorded.

Results

The results are given in Table 248. At lengths of 10mm or longer, explants of Rhododendron concinnum survived, but in shorter explants, survival was decreased. However, explants of Rhododendron ponticum survived when 5mm or longer. Stems of R. ponticum have a greater diameter (mean diameter of 20 stems = 2.84mm) than those of R. concinnum (mean stem diameter = 1.1mm) and therefore, volumes of the stems were measured for comparison. The volume of explants was determined by measuring the volume of water displaced when ten explants were added to it. The minimum volume for survival on Anderson (1975) (A1) medium was the same for both species tested (Table 248).

Table 248.

Explant size in relation to survival

Number survived (of 3 replicate explants)

	R. concinnum		R. ponticum	
length (mm)	mean volume (cc)	number survived	mean volume	number survived
2.5	0.0064	0	0.025	2
5.0	0.012	1	0.05	3
10.0	0.028	3	0.10	3
20.0	0.052	3	0.20	3
40.0	0.105	3	0.40	3
80.0	0.216	3	0.80	3

11.4 STERILIZATION OF EXPLANTS

Different sterilants and sterilization times have been used by different workers depending on species and physiological condition of the explants. It was therefore necessary to ascertain the most appropriate sterilization treatments for the species used in the experiments described in this thesis.

Method

The species tested were Rhododendron concinnum and Arctostaphylos uva-ursi. Explants from woody and non-woody (actively elongating with little secondary growth) shoots were tested a) for the effectiveness of the sterilants in eliminating contaminants and b) for their sensitivity to concentration of sterilants.

The sterilants used were 1) ethanol at concentrations of 30, 50 and 70%; 2) sodium hypochlorite at concentrations of 0.1, 0.5, 2.5, and 5.0%; 3) water as a control. A few drops of detergent were added as a wetting agent. Woody explants were pretreated with antioxidant (see Appendix 11.5).

Duration of sterilization was 0.5, 1, 2, 5, 10, 20, 30, 40, 50, or 60 minutes. There were three replicates per treatment.

After sterilization, explants were rinsed twice in sterile distilled water and incubated on a mediium containing sucrose. Survival andcontamination were assessed at 10 days.

Results

Results are given in Tables 249 to 256. There was little difference between the two species in their response to the different sterilization treatments. In non-woody explants survival was greatest and contamination least after sterilization with sodium hypochlorite at a concentration of 0.5% for 10 to 20 minutes. For woody explants, a stronger sterilization treatment was necessary. In the water control, all explants survived but all were contaminated. Considerable damage occurred in explants treated with ethanol; although some survived, all exhibited some discoloration of the tissue.

Table 249.

Survival and contamination of Rhododendron concinnum non-woody explants after sterilization with ethanol

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	ethanol concentration 30%	50%	70%
0.5	3 (3)	1 (3)	1 (3)
1	1 (3)	0 (1)	0 (1)
2	0 (1)	0 (1)	0 (0)
5	0 (0)	0 (0)	0 (0)
10	0 (0)	0 (0)	0 (0)
20	0 (0)	0 (0)	0 (0)
30	0 (0)	0 (0)	0 (0)
40	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)

Table 250.

Survival and contamination of Rhododendron concinnum woody explants after sterilization with ethanol

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	ethanol concentration 30%	50%	70%
0.5	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (2)	2 (1)
2	3 (2)	1 (1)	0 (0)
5	1 (0)	0 (0)	0 (0)
10	0 (0)	0 (0)	0 (0)
20	0 (0)	0 (0)	0 (0)
30	0 (0)	0 (0)	0 (0)
40	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)

Table 251.

Survival and contamination of Arctostaphylos uva-ursi non-woody explants after sterilization with ethanol

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	ethanol concentration 30%	50%	70%
0.5	3 (3)	3 (3)	1 (3)
1	2 (2)	0 (1)	0 (1)
2	0 (1)	0 (1)	0 (0)
5	0 (0)	0 (0)	0 (0)
10	0 (0)	0 (0)	0 (0)
20	0 (0)	0 (0)	0 (0)
30	0 (0)	0 (0)	0 (0)
40	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)

Table 252.

Survival and contamination of Arctostaphylos uva-ursi woody explants after sterilization with ethanol

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	ethanol concentration 30%	50%	70%
0.5	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (2)	1 (2)
2	1 (2)	1 (2)	0 (1)
5	0 (0)	0 (0)	0 (0)
10	0 (0)	0 (0)	0 (0)
20	0 (0)	0 (0)	0 (0)
30	0 (0)	0 (0)	0 (0)
40	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)

Table 253.

Survival and contamination Arctostaphylos uva-ursi non-woody explants after sterilization with sodium hypochlorite

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	concentration of sodium hypochlorite			
	0.1%	0.5%	2.5%	5%
0.5	3 (3)	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (3)	3 (3)	3 (3)
2	3 (3)	3 (3)	3 (3)	3 (3)
5	3 (3)	3 (3)	3 (1)	1 (1)
10	3 (3)	3 (0)	2 (0)	0 (0)
20	3 (1)	3 (0)	1 (0)	0 (0)
30	3 (1)	1 (0)	0 (0)	0 (0)
40	1 (0)	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)	0 (0)

Table 254.

Survival and contamination Arctostaphylos uva-ursi woody explants after sterilization with sodium hypochlorite

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	concentration of sodium hypochlorite			
	0.1%	0.5%	2.5%	5%
0.5	3 (3)	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (3)	3 (3)	3 (3)
2	3 (3)	3 (3)	3 (3)	3 (3)
5	3 (3)	3 (3)	3 (1)	1 (1)
10	3 (3)	3 (0)	2 (0)	0 (0)
20	3 (2)	3 (2)	3 (2)	1 (1)
30	3 (2)	3 (1)	2 (1)	1 (0)
40	3 (1)	3 (0)	1 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)	0 (0)

Table 255.

Survival and contamination of Rhododendron concinnum non-woody explants after sterilization with sodium hypochlorite

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	concentration of sodium hypochlorite			
	0.1%	0.5%	2.5%	5%
0.5	3 (3)	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (3)	3 (3)	3 (3)
2	3 (3)	3 (3)	3 (3)	3 (3)
5	3 (3)	3 (3)	3 (2)	2 (0)
10	3 (3)	3 (0)	3 (0)	1 (0)
20	3 (2)	3 (0)	2 (0)	1 (0)
30	1 (0)	0 (0)	0 (0)	0 (0)
40	0 (0)	0 (0)	0 (0)	0 (0)
50	0 (0)	0 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)	0 (0)

Table 256.

Survival and contamination of Rhododendron concinnum woody explants after sterilization with sodium hypochlorite

Number survived (of three replicates) and number of cultures contaminated (figure given in brackets)

duration of treatment (minutes)	concentration of sodium hypochlorite			
	0.1%	0.5%	2.5%	5%
0.5	3 (3)	3 (3)	3 (3)	3 (3)
1	3 (3)	3 (3)	3 (3)	3 (3)
2	3 (3)	3 (3)	3 (3)	3 (3)
5	3 (3)	3 (3)	3 (3)	1 (2)
10	3 (3)	3 (3)	2 (3)	1 (1)
20	3 (3)	3 (3)	3 (2)	1 (1)
30	3 (2)	3 (2)	3 (1)	1 (0)
40	3 (1)	3 (0)	1 (0)	0 (0)
50	2 (0)	1 (0)	0 (0)	0 (0)
60	0 (0)	0 (0)	0 (0)	0 (0)

11.5 PRE-TREATMENT OF EXPLANTS WITH ANTIOXIDANTS

Woody explants were susceptible to tissue browning after excision from the parent plant. In many cases, the browning resulted in death of the tissue. Such browning is often due to the oxidation of phenolic compounds, and thereforetreatments were given to try to improve survival of the explants.

Method

Shoot tip explants of Rhododendron concinnum (ten explants per treatment) were soaked in the following antioxidant solutions:-

1) ascorbic acid (50 mg l^{-1}) + citric acid (75 mg l^{-1}) (X 1/2)
2) ascorbic acid (100 mg l^{-1}) + citric acid (150 mg l^{-1}) (X 1)
3) ascorbic acid (200 mg l^{-1}) + citric acid (300 mg l^{-1}) (X 2)
4) ascorbic acid (100 mg l^{-1})
5) distilled water (control)
6) no soaking treatment (control)

Explants were soaked for 30, 60, 120 or 180 minutes and then were transferred to the surface of an agar medium and examined for browning after an incubation period of 48 hours.

Results

Results are given in Table 257. The only treatments which improved survival over the control were a one hour soak in X 1 concentration and a 30 minute soak in X 2 concentration of ascorbic acid and citric acid as specified. Prolonged soaking in any solution was detrimental to survival and resulted in increased contamination of explants.

Table 257.

Survival of woody explants of Rhododendron concinnum after pre-treatment with antioxidants

Number survived (of ten replicates) after 48 hours

antioxidant solution	duration of treatment (minutes)				
	0	30	60	120	180
X 1/2	7	4	3	0	0
X 1		5	10	7	2
X 2		9	4	2	0
ascorbic		4	3	0	0
water		4	1	0	0

11.6 SOURCES OF TRADE MARKED CHEMICALS AND EQUIPMENT

Clorox (bleach)

The Clorox Company,
Oakland,
California 94612, U.S.A.

Millipore (filters)

Millipore Filter Corporation,
Bedford,
Massachusetts, U.S.A.

Parafilm (sealing film)

Dixie / Marathon,
Greenwich,
Connecticut 06830, U.S.A.

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